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TITLE OF INVENTION

RECOMBINANT PROTEIN CONTAINING A C-TERMINAL FRAGMENT OF PLASMODIUM MSP-1

APPLICANT(S) FOR DO/EO/US

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Applicant herewith submits to the United States Designated/Elected Office (DO/EO/US) the following items and other information:

1. ☒ This is a **FIRST** submission of items concerning a filing under 35 U.S.C. 371.
2. ☐ This is a **SECOND** or **SUBSEQUENT** submission of items concerning a filing under 35 U.S.C. 371.
3. ☒ This is an express request to begin national examination procedures (35 U.S.C. 371(f)) at any time rather than delay examination until the expiration of the applicable time limit set in 35 U.S.C. 371(b) and PCT Articles 22 and 39(1).
4. ☒ A proper Demand for International Preliminary Examination was made by the 19th month from the earliest claimed priority date.
5. ☒ A copy of the International Application as filed (35 U.S.C. 371 (c) (2))
  - a. ☐ is transmitted herewith (required only if not transmitted by the International Bureau).
  - b. ☒ has been transmitted by the International Bureau.
  - c. ☐ is not required, as the application was filed in the United States Receiving Office (RO/US).
6. ☒ A translation of the International Application into English (35 U.S.C. 371(c)(2)).
7. ☒ A copy of the International Search Report (PCT/ISA/210).
8. ☒ Amendments to the claims of the International Application under PCT Article 19 (35 U.S.C. 371 (c)(3))
  - a. ☐ are transmitted herewith (required only if not transmitted by the International Bureau).
  - b. ☐ have been transmitted by the International Bureau.
  - c. ☐ have not been made; however, the time limit for making such amendments has NOT expired.
  - d. ☒ have not been made and will not be made.
9. ☐ A translation of the amendments to the claims under PCT Article 19 (35 U.S.C. 371(c)(3)).
10. ☐ An oath or declaration of the inventor(s) (35 U.S.C. 371 (c)(4)).
11. ☐ A copy of the International Preliminary Examination Report (PCT/IPEA/409).
12. ☐ A translation of the annexes to the International Preliminary Examination Report under PCT Article 36 (35 U.S.C. 371 (c)(5)).

## Items 13 to 18 below concern document(s) or information included:

13. ☐ An Information Disclosure Statement under 37 CFR 1.97 and 1.98.
14. ☐ An assignment document for recording. A separate cover sheet in compliance with 37 CFR 3.28 and 3.31 is included.
15. ☐ A **FIRST** preliminary amendment.  
A **SECOND** or **SUBSEQUENT** preliminary amendment.
16. ☐ A substitute specification.
17. ☐ A change of power of attorney and/or address letter.
18. ☐ Certificate of Mailing by Express Mail
19. ☒ Other items or information:

Request for Consideration of Documents Cited in International Search Report

32 Sheets of Drawings

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NOTICE OF PRIORITY



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**RECOMBINANT PROTEIN CONTAINING A C-TERMINAL FRAGMENT**  
**OF PLASMODIUM MSP-1**

The invention relates to novel active principles for vaccines derived from the major surface protein in merozoite forms of a *Plasmodium* which is infectious for mammals, especially humans, more generally termed MSP-1.

MSP-1 has already been the subject of a number of studies. It is synthesised in the schizont stage of *Plasmodium* type parasites, in particular *Plasmodium falciparum*, and is expressed in the form of one of the major surface constituents of merozoites both in the hepatic stage and in the erythrocytic stage of malaria (1, 2, 3, 4). Because of the protein's predominant character and conservation in all known *Plasmodium* species, it has been suggested that it could be a candidate for constituting anti-malarial vaccines (5, 6).

The same is true for fragments of that protein, particularly the natural cleavage products which are observed to form, for example during invasion by the parasite into erythrocytes of the infected host. Among such cleavage products are the C-terminal fragment with a molecular weight of 42 kDa (7, 8) which is itself cleaved once more into an N-terminal fragment with a conventional apparent molecular weight of 33 kDa and into a C-terminal fragment with a conventional apparent molecular weight of 19 kDa (9) which remains normally fixed to the parasite membrane after the modifications carried out on it, via glycosylphosphatidylinositol (GPI) groups (10, 11).

It is also found at the early ring stage of the intraerythrocytic development cycle (15, 16), whereby the observation was made that the 19 kDa fragment could play a role which is not yet known, but which is doubtless essential in re-invasive processes. This formed the basis for hypotheses formed in the past that that protein could constitute a particularly effective target for possible vaccines.

It should be understood that the references frequently made below to the p42 and p19 proteins from a certain type of *Plasmodium* are

understood to refer to the corresponding C-terminal cleavage products of the MSP-1 protein of that *Plasmodium* or, by extension, to products containing substantially the same amino acid sequences, obtained by genetic recombination or by chemical synthesis using conventional techniques, for example using the "Applied System" synthesiser, or by "Merrifield" type solid phase synthesis. For convenience, references to "recombinant p42" and "recombinant p19" refer to "p42" and "p19" obtained by techniques comprising at least one genetic engineering step.

Faced with the difficulty of obtaining large quantities of parasites for *P. falciparum* and the impossibility of cultivating *P. vivax in vitro*, it has become clear that the only means of producing an anti-malaria vaccine is to resort to techniques which use recombinant proteins or peptides. However, MSP-1 is very difficult to produce whole because of its large size of about 200 kDa, a fact which has led researchers to study the C-terminal portion, the (still unknown) function of which is probably the more important.

Recombinant proteins concerning the C-terminal portion of the *P. falciparum* MSP-1 which have been produced and tested in the monkey (12, 40, 41) are:

- a p19 fused with a glutathione-S-transferase produced in *E. coli* (40);
- a p40 fused with a glutathione-S-transferase produced in *E. coli* (12);
- a p19 fused with a polypeptide from a tetanic anatoxin and carrying auxiliary T cell epitopes produced in *S. cerevisiae* (12);
- a p42 produced in a baculovirus system (41).

A composition containing a p19 protein fused with a glutathione-S-transferase produced in *E. coli* combined with alum or liposomes did not exhibit a protective effect in any of six vaccinated *Aotus nancymai* monkeys (40).

A composition containing a p42 protein fused with a glutathione-S-transferase produced in *E. coli* combined with a Freund complete adjuvant did not exhibit a protective effect in two types of *Aotus* monkeys (A.

*nancymai* and *A. vociferans*) when administered to them. The p19 protein produced in *S. cerevisiae* exhibited a protective effect in two *A. nancymai* type *Aotus* monkeys (12). In contrast, there was no protective effect in two *A. vociferans* type *Aotus* monkeys.

Some researchers (Chang et al.) have also reported immunisation tests carried out in the rabbit using a recombinant p42 protein produced in a baculovirus system and containing one amino acid sequence in common with *P. falciparum* (18). Thus these latter authors indicate that in the rabbit that recombinant p42 behaves substantially in the same way as the entire recombinant MSP-1 protein (gp195). This p42 protein in combination with a Freund complete adjuvant has been the subject matter of a vaccination test in a non-human primate susceptible to infection by *P. falciparum*, *Aotus*, *lemurinus grisemembra* (40). The results showed that 2 of 3 animals were completely protected and the third, while exhibiting a parasitemia which resembled that of the controls, had a longer latent period. It is nevertheless risky to conclude to a protective nature in man of the antibodies thus induced against the parasites themselves. It should be remembered that there are currently no very satisfactory experimental models in the primate for *P. vivax* and *P. falciparum*. The *Saimiri* model, developed for *P. falciparum* and *P. vivax*, and the *Aotus* model for *P. falciparum*, are artificial systems requiring the parasite strains to be adapted and often requiring splenectomy of the animals to obtain significant parasitemia. As a result, the vaccination results from such models can only have a limited predictive value for man.

In any event, what the real vaccination rate would be which could possibly be obtained with such recombinant proteins is also questionable, bearing in mind the discovery - reported below - of the presence in p42s from *Plasmodiums* of the same species, and more particularly in the corresponding p33s, of hypervariable regions which would in many cases render uncertain the immunoprotective efficacy of antibodies induced in

individuals vaccinated with a p42 from a *Plasmodium* strain against an infection by other strains of the same species (13).

It can even be assumed that the high polymorphism of the N-terminal portion of p42 plays a significant role in immune escape, often observed for that type of parasite.

The aim of the present invention is to produce vaccinating recombinant proteins which can escape these difficulties, the protective effect of which is verifiable in genuinely significant experimental models or even directly in man.

More particularly, the invention provides vaccinating compositions against a *Plasmodium* type parasite which is infectious for man, containing as an active principle a recombinant protein which may or may not be glycosylated, whose essential constituent polypeptide sequence is:

- either that of a 19 kilodalton (p19) C-terminal fragment of the surface protein 1 of the merozoite form (MSP-1 protein) of a *Plasmodium* type parasite which is infectious for man, said C-terminal fragment remaining normally anchored to the parasite surface at the end of its penetration phase into human erythrocytes in the event of an infectious cycle;
- or that of a portion of that fragment which is also capable of inducing an immune response which can inhibit *in vivo* parasitemia due to the corresponding parasite;
- or that of an immunologically equivalent peptide of said p19 fragment or said portion of that fragment; and

said recombinant protein further comprises conformational epitopes which are unstable in a reducing medium and which preferably constitute the majority of the epitopes recognised by human antisera formed against the corresponding *Plasmodium*.

The presence of such conformational epitopes could play an important role in the protective efficacy of the active principle of the vaccines. They are particularly found in the active principles which exhibit

the other characteristics defined above, when they are produced in a baculovirus system. If needs be, it is mentioned below that the expression "baculovirus vector system" means the ensemble constituted by the baculovirus type vector itself and the cell lines, in particular cells of insects transfectable by a baculovirus modified by a sequence to be transferred to these cell lines resulting in expression of that transferred sequence. Preferred examples of these two partners in the baculovirus system have been described in the article by Longacre et al. (19). The same system was used in the examples below. It goes without saying, of course, that variations in the baculovirus and in the cells which can be infected by the baculovirus can be used in place of those selected.

In particular, the recombinant protein is recognised by human antisera formed against the corresponding *Plasmodium* or against a homologous *Plasmodium* when it is in its non reduced state or in a reduced non irreversible state, but is not recognised or is only recognised to a slight extent by these same antisera when it is irreversibly reduced.

The unstable character of these conformational epitopes in a reducing medium can be demonstrated by the test described below in the examples, in particular in the presence of  $\beta$ -mercaptoethanol. Similarly, the examples below describe the experimental conditions applicable to obtain irreversible reduction of the proteins of the invention.

From this viewpoint, the recombinant protein produced by Longacre et al. (14) can be used in such compositions. It should be remembered that S. Longacre et al. succeeded in producing a recombinant p19 from the MSP-1 of *P. vivax* in a baculovirus vector system containing a nucleotide sequence coding for the p19 of *Plasmodium vivax*, in particular by transfecting cultures of insect cells [*Spodoptera frugiperda* (Sf9) line] with baculovirus vectors containing, under the control of the polyhedrin promoter, a sequence coding for the peptide sequences defined below, with the sequences being placed in the following order in the baculovirus vector used:

- a 35 base pair 5' terminal fragment of the polyhedrin signal sequence, in which the methionine codon for initiating expression of this protein had been mutated (to ATT);
- a 5'-terminal nucleotide fragment coding for a 32 amino acid peptide corresponding to the N-terminal portion of MSP-1, including the MSP-1 signal peptide;
- either a nucleotide sequence coding for p19, or a sequence coding for the p42 of the MSP-1 protein of *Plasmodium vivax*, depending on the case, these sequences also being provided with ("anchored" forms) or deprived of (soluble forms) 3' end regions of these nucleotide sequences, whose end C-terminal expression products are reputed to play an essential role in anchoring the final p19 protein to the parasite membrane;
- 2 TAA stop codons.

For p42, the sequences derived from the C-terminal region of MSP-1 extend consequently from amino acid Asp 1325 to amino acid Leu 1726 (anchored form) or to amino acid Ser 1705 (soluble form) and for p19, the sequences extend from amino acid Ile 1602 to amino acid Leu 1726 (anchored form) or to amino acid Ser 1705 (soluble form) it being understood that the complete amino acid sequences of p42 and p19, whose initial and terminal amino acids have been indicated above follow from the gene of the Belem isolate of *P. vivax* which has been sequenced (20).

Similar results were obtained using, in the same vector systems, nucleotide sequences coding for the p42 and p19 of *Plasmodium cynomolgi*. The interest in *P. cynomolgi* is twofold: it is a parasitic species very close to *P. vivax* which is infectious for the macaque. It can also infect man. Further, access to the natural hosts of *P. cynomolgi*, rhesus monkeys and toque macaques, is also possible, to test the efficacy of the protection of MSP-1 from *P. cynomolgi* in natural systems. The rhesus monkey is



considered to be one of the most representative species for immune reactions in man.

In particular, excellent results have been obtained in vaccination tests carried out using the toque macaque with two recombinant polypeptides: soluble p42 and, in particular, soluble p19 derived from *P. cynomolgi*, respectively produced in a baculovirus system and purified on an affinity column with monoclonal antibodies recognising the corresponding regions of the native MSP-1 protein. The following observations were made: six monkeys immunised with only p19 (three monkeys) and the p19 and p42 together (three monkeys) all exhibited practically sterile immunity after challenge infection. The results obtained in the three monkeys immunised with p42 were less significant. Two of them were as above, but since the third exhibited a lower parasitemia than the controls immunised with a PBS buffer in the presence of Freund adjuvant (3 monkeys) or not immunised (3 monkeys), it was less clear.

A second challenge infection showed that the monkeys which had received p19 alone were protected for at least six months. A second vaccination test with p19 in combination with alum in this system (toque macaque *P. cynomolgi*) exhibited significant protection for 2 of the 3 monkeys. This is the first time that MSP-1 or another recombinant antigen has demonstrated a protective effect in the presence of alum (42).

The particularly effective test results carried out with the macaque with recombinant polypeptides produced in a baculovirus system using a recombinant p19 from *P. cynomolgi* showed that recombinant polypeptides respectively containing recombinant p19s from other *Plasmodiums* must behave in the same manner. They are more meaningful for malaria in man than the results from tests carried out with *P. vivax* or *P. falciparum* in their "artificial hosts".

Baculovirus recombinant proteins derived from a C-terminal MSP-1 portion (p19) have a very significant antimalarial protective effect in a

natural system, which constitutes the most representative model for evaluating the protective effect of MSP-1 for man.

The protective effect obtained can be further improved if the p19 form is deprived of the hypervariable region of the N-terminal portion of p42, the effect of which can be deleterious in natural situations in which the vaccinated subject is confronted by a great deal of polymorphism. Further, p19 appears to possess specific epitopes which are not present in p42.

The 19 kDa C-terminal fragment, the sequence of which is present in the active principle of the vaccine, can be limited to the sequence for the p19 itself, in the absence of any polypeptide sequence normally upstream of the p19 sequence in the corresponding MSP-1 protein. Clearly, though, the essential constituent polypeptide sequence of the active principle can also comprise a polypeptide sequence for the C-terminal side belonging to the 33 kDa (p33) N-terminal fragment still associated with the p19 in the corresponding p42, before natural cleavage of the latter, if the presence of this fragment does not modify the immunological properties of the active principle of the vaccine. As will be seen below, in particular in the description of the examples, the C-terminal sequences of the p33 in various strains of the same species of *Plasmodium* (see the C-terminal portion of the peptide sequences of "region III" in Figure 4) also have a degree of homology or substantial conservation of the sequence, for example of the order of at least 80%, in different varieties of *Plasmodiums* which are infectious for man, such that they do not fundamentally modify the vaccinating properties of the active principle (the sequence of which corresponds to region IV in Figure 4), in particular using the hypothesis which follows from this figure; that the presumed cleavage site between the p19 and region III of the p33 is located between the leucine and asparagine residues in a particularly well conserved region (LNVQTQ).

Normally the C-terminal polypeptide sequence of the p33, when it is present, comprises less than 50 amino acid residues, or even less than 35, preferably less than 10 amino acid residues.

In contrast, the essential constituent polypeptide sequence of the active principle of the vaccine need not comprise all of the sequence coding for p19, naturally providing that the latter retains the ability to induce antibodies which protect against the parasite. In particular, the molecular weight of the "fragment portion" is 10 to 25 kDa, in particular 10 to 15 kDa. Preferably, this polypeptide fragment portion contains at least one of the two EGF (Epidermal Growth Factor) regions.

Clearly, the skilled person could distinguish between active fragments and those which would no longer be so, in particular experimentally by producing modified vectors containing inserts with different lengths originating from the p19, respectively isolated from the fragments obtained from the sequence coding for p19, by reaction with appropriate restriction enzymes, or by exonucleolytic enzymes which would be kept in contact with the fragment coding for p19 for differing periods; the capacity of the expression products from these inserts in the corresponding eukaryotic cells, in particular in insect cells, transformed by the corresponding modified vectors, to exert a protective effect can then be tested, in particular under the experimental conditions which are described below in the examples. In particular, the expression products of these inserts must be able to inhibit a parasitemia induced *in vivo* by the corresponding whole parasite.

Thus, the invention includes all vaccinating compositions in which the essential constituent polypeptide sequence of the active principle is constituted by a peptide which can induce a cellular and/or humoral type immunological response equivalent to that produced by p19 or a fragment as defined above, provided that the addition, deletion or substitution in the sequence of certain amino acids by others would not cause a large modification of the capacity of the modified peptide - hereinafter termed the "immunologically equivalent peptide" - to inhibit said parasitemia.

The p19 fragment can naturally also be associated at the N-terminal side or the C-terminal side or via a peptide bond to a further plasmoidal protein fragment having a vaccinating potential (such as Duffy binding protein

from *P. vivax* (29) or EBA-175 from *P. falciparum* (30) and (31), one region of which is specifically rich in cysteine), provided that its capacity to inhibit parasitemia normally introduced *in vivo* by the corresponding parasite is not altered but is amplified.

5 Upstream of the N-terminal end of p19, the fragment coding for p19 or a portion thereof can also contain a peptide sequence which is different again, for example a C-terminal fragment of the signal peptide used, such as that for the MSP-1 protein. This sequence preferably comprises less than 50 amino acids, for example 10 to 40 amino acids.

10 These observations pertain in similar fashion to the p19s from other *Plasmodium*, in particular *P. falciparum*, the dominant species of the parasites, responsible for one of the most serious forms of malaria.

However, the techniques summarised above for producing a recombinant p19 from *P. vivax* or *P. cynomolgi* in a baculovirus system are  
15 difficult to transpose unchanged to producing a recombinant p19 of *P. falciparum* in a satisfactory yield, if only to obtain appreciable quantities which will allow immunoprotective tests to be carried out.

The invention also provides a process which overcomes this problem to a large extent. It also becomes possible to obtain much higher yields of *P. falciparum* p19 - and other *Plasmodiums* where similar difficulties are  
20 encountered - using a synthetic nucleotide sequence substituting the natural nucleotide sequence coding for the p19 of *Plasmodium falciparum* in an expression vector of a baculovirus system, this synthetic nucleotide sequence coding for the same p19, but being characterized by a higher  
25 proportion of G and C nucleotides than in the natural nucleotide sequence.

In other words, the invention follows from the discovery that expression of a nucleotide sequence coding for a p19 in a baculovirus system is apparently linked to an improved compatibility of successive codons in the nucleotide sequence to express with the "cellular machinery"  
30 of the host cells transformable by the baculovirus, in the manner of that observed for the natural nucleotide sequences normally contained in these

baculovirus and expressed in the infected host cells; hence the poor expression, or even total absence of expression of a native *P. falciparum* nucleotide sequence; hence also a possible explanation of the more effective expression observed by Longacre et al. (14) for the p19 of *P. vivax* in a baculovirus system and, as the inventors have also shown, of the *P. cynomolgi* sequence from corresponding native p19 nucleotide sequences, because of their relatively much higher amounts of G and C nucleotides than those of the native nucleotide sequences coding for the p19 of *P. falciparum*.

The invention thus more generally provides a recombinant baculovirus type modified vector containing, under the control of a promoter contained in said vector and able to be recognised by cells transfectable by said vector, a first nucleotide sequence coding for a signal peptide exploitable by a baculovirus system, characterized by a second nucleotide sequence downstream of the first, also under the control of the promoter and coding for the peptide sequence:

- either of a 19 kilodalton (p19) C-terminal fragment of the surface protein 1 of the merozoite form (MSP-1 protein) of a *Plasmodium* type parasite other than *Plasmodium vivax* which is infectious for man, said C-terminal fragment remaining normally anchored to the parasite surface at the end of its penetration phase into human erythrocytes in the event of an infectious cycle;
- or of a portion of that peptide fragment provided that the expression product from the second sequence in a baculovirus system is also capable of inducing an immune response which can inhibit *in vivo* parasitemia due to the corresponding parasite;
- or of an immunologically equivalent peptide of said C-terminal peptide fragment (p19) or said peptide fragment portion by addition, deletion or substitution of amino acids not resulting in a large modification of the capacity of said immunologically equivalent peptide to induce a cellular

and/or humoral type immunological response similar to that produced by said p19 peptide fragment or said portion of said fragment; and said nucleotide sequence having, if necessary, a G and C nucleotide content in the range 40% to 60%, preferably at least 50%, of the totality of the nucleotides from which it is constituted. This sequence can be obtained by constructing a synthetic gene in which the natural codons have been changed for codons which are rich in G/C without modifying their translation (maintaining the peptide sequence).

The nucleotide sequence, provided by a synthetic DNA, may have at least 10% of modified codons with respect to the natural gene sequence or cDNA while retaining the characteristics of the natural translated sequence, i.e., maintaining the amino acid sequence.

It is not excluded that this G and C nucleotide content could be further increased provided that the modifications resulting therefrom as to the amino acid sequence of the recombinant peptide - or immunologically equivalent peptide - produced do not result in a loss of immunological properties, or protective properties, of the recombinant proteins formed, in particular in the tests which will be described below.

These observations naturally apply to other *Plasmodium* which are infectious for man, in particular those where the native nucleotide sequences coding for corresponding p19s would have T and A nucleotide contents which are poorly compatible with effective expression in a baculovirus system.

The sequence coding for the signal used can be that normally associated with the native sequence of the *Plasmodium* concerned. But it can also originate from another *Plasmodium*, for example *P. vivax* or *P. cynomolgi* or another organism if it can be recognised as a signal in a baculovirus system.

The sequence coding for p19 or a fragment thereof in the vector under consideration is, in one case, deprived of the anchoring sequence of the native protein to the parasite from which it originates, in which case the

expressed protein is generally excreted into the culture medium (soluble form). It is also remarkable in this respect that under the conditions of the invention, the soluble and anchored forms of the recombinant proteins produced, in particular when they are from *P. falciparum* or *P. cynomolgi* or  
5 *P. vivax*, tend to form oligomers, this property possibly being at the origin of the increased immunogenicity of the recombinant proteins formed.

The invention also concerns vectors in which the coding sequence contains the terminal 3'end sequence coding for the hydrophobic C'-terminal end sequence of the p19 which is normally implicated in the  
10 induction of anchoring the native protein to the cell membrane of the host in which it is expressed. This 3'-terminal end region can also be heterologous as regards the sequence coding for the soluble p19 portion, for example corresponding to the 3'-terminal sequence from *P. vivax* or from another organism when it codes for a sequence which anchors the whole of the  
15 recombinant protein produced to the cell membrane of the host of the baculovirus system used. An example of such anchoring sequences is the GPI of the CD59 antigen which can be expressed in the cells of *Spodoptera frugiperda* (32) type insects or the GPI of a CD14 human protein (33).

20 The invention also, naturally, concerns recombinant proteins, these proteins comprising conformational epitopes recognised by human serums formed against the corresponding *Plasmodium*.

In general, the invention also concerns any recombinant protein of the type indicated above, provided that it comprises conformational  
25 epitopes such as those produced in the baculovirus system, in particular those which are unstable in a reducing medium.

The invention also, naturally, concerns said recombinant proteins, whether they are in their soluble form or in the form provided with an anchoring region, in particular to cellular hosts used in the baculovirus  
30 system.

The invention also encompasses oligomers spontaneously produced in the baculovirus systems used or produced *a posteriori*, using conventional protein oligomerisation techniques. The most commonly used technique involves glutaraldehyde. However, any conventional system for bridging between the respective amine and carboxyl functions in proteins can be used. As an example, any of the techniques described in European patent application EP-A-0 602 079 can be used.

The term "oligomer" means a molecule containing 2 to 50 monomer units, each of the monomer units containing p19 or a fragment thereof, as defined above, capable of forming an aggregate. The invention also encompasses any conjugation product between a p19 or a p19 fragment as defined above, and a carrier molecule - for example a polylysine-alanine - for use in producing vaccines, via bonds which are covalent or otherwise. The vaccinating compositions using them also form part of the invention.

The invention still further concerns vaccine compositions using these oligomeric or conjugated recombinant proteins, including proteins from *Plasmodium vivax*, these observations also extending to oligomers of these recombinant proteins.

The invention also encompasses compositions in which the recombinant proteins defined above are associated with an adjuvant, for example an alum. Recombinant proteins containing the C-terminal end region allowing them to anchor to the membrane of the cells in which they are produced are advantageously used in combination with lipids which can form liposomes appropriate to the production of vaccines. Without being limiting, lipids described, for example, in the publication entitled "Les liposomes aspects technologique, biologique et pharmacologique" [Liposomes: technological, biological and pharmacological aspects] by J. Delattre et al., INSERM, 1993, can be used.

The presence of the anchoring region in the recombinant protein, whether it is a homologous or heterologous anchoring region as regards the vaccinating portion proper, encourages the production of cytophilic



antibodies, in particular IgG<sub>2a</sub> and IgG<sub>2b</sub> type in the mouse which could have a particularly high protective activity, so that associating the active principles of the vaccines so constituted with adjuvants other than the lipids used to constitute the liposome forms could be dispensed with. This amounts to a major advantage, since liposomes can be lyophilised under conditions which enable them to be stored and transported, without the need for chains of cold storage means.

Other characteristics of the invention will become clear from the following description of examples of recombinant proteins of the invention and the conditions under which they can be produced. These examples are not intended to limit the scope of the invention.

**Description of the construction of PfMSP1<sub>p19</sub>S (soluble) (soluble p19 from *P. falciparum*)**

The recombinant construction PfMSP1<sub>p19</sub>S contains the DNA corresponding to 8 base pairs of the leader sequence and the first 32 amino acids of the MSP-1 of *Plasmodium vivax* from Met<sub>1</sub> to Asp<sub>32</sub> (Belem isolate; Del Portillo et al., 1991, P. N. A. S., 88, 4030) followed by GluPhe due to the EcoR1 site connecting the two fragments. This is followed by the synthetic gene described in Figure 1, coding the *Plasmodium falciparum* MSP1<sub>p19</sub> from Asn<sub>1613</sub> to Ser<sub>1705</sub> (Uganda-Palo Alto isolate; Chang et al., 1988, Exp. Parasitol., 67, 1). The construction is terminated by two TAA stop codons. This construction gave rise to a recombinant protein which was secreted in the culture supernatant from infected cells.

In the same manner and for comparison, a recombinant construction was produced under conditions which were similar to those used to produce the p19 above, but working with a coding sequence consisting of a direct copy of the corresponding DNA of the *P. falciparum* strain (FUP) described by Chang et al., Exp. Parasit. 67,1; 1989. The natural gene copy (from asparagine 1613 to serine 1705) was formed from the native gene by PCR.

**Figure 1A** shows the sequences of both the synthetic gene (Bac19) and the "native gene" (PF19).

It can be seen that 57 codons of the 93 codons of the native sequence coding for the p19 from *P. falciparum* were modified (the third nucleotide in 55 of them and the first and third nucleotides in the other 2 codons). New codons were added to the 5' end to introduce the peptide signal under the conditions indicated above and to introduce an EcoRI site for cloning, and similarly two stop codons were added which were not present in the *P. falciparum* p19 to obtain expression termination signals. The individual letters placed above successive codons correspond to the respective successive amino acids. Asterisks (\*) show the stop codons. Vertical lines indicate the nucleotides which are the same in the two sequences

**Description of the PfMSP1<sub>p19</sub>A construction (anchored GPI) (anchored p19 of *P. falciparum*)**

The PfMSP1<sub>p19</sub>A construction had the characteristics of that above except that the synthetic sequence (Figure 1B) codes for the MSP1<sub>p19</sub> of *Plasmodium falciparum* (Uganda-Palo Alto isolate) from Asn<sub>1613</sub> to Ile<sub>1726</sub> followed by two TAA stop codons. This construction gave rise to a recombinant protein which was anchored in the plasma membrane of infected cells by a glycosyl phosphatidyl inositol (GPI) type structure.

Figure 1C represents the PfMSP1<sub>p19</sub>S recombinant protein sequence before cutting out the signal sequence.

Figure 1D represents the PfMSP1<sub>p19</sub>S recombinant protein sequence after cutting out the signal sequence.

The amino acids underlined in Figures 1C and 1D originate from the EcoRI site used to join the nucleotide sequences derived from the N-terminal portion of the MSP-1 of *P. vivax* (with signal sequence) and the MSP-1<sub>p19</sub> of *P. falciparum*.

Figure 2 - The soluble recombinant PfMSP1<sub>p19</sub> antigen purified by Immunoaffinity was analysed by immunoblot using SDS-PAGE in the presence (reduced) or absence (non reduced) of β-mercaptoethanol. Samples were charged onto gel after heating to 95°C in the presence of

2% SDS. Under these conditions only covalent type bonds (disulphide bridges) can resist disaggregation. The left hand blot was revealed with a monoclonal antibody which reacted with a linear epitope of natural p19. The right hand blot was revealed with a mixture of 13 human antisera originating from subjects with acquired immunity to malaria due to *Plasmodium falciparum*. These results show that the recombinant baculovirus molecule can reproduce conformational epitopes in the form of a polymer the majority of which are recognised by human antiserum.

**Figure 2B: Immunoblot analysis with human antiserum of recombinant purified MSP-1 p19 from *P. vivax* and *P. cynomolgi* under non reduced (NR), reduced only in the charging medium (R) and Irreversibly reduced (IR) conditions:**

This work was based on the idea that the baculovirus expression system correctly reproduced the conformational epitopes present *in vivo* on the C-terminal portion of MSP-1 in large amounts. The best means of measuring this property (which may be the only possible means in the absence of native purified proteins corresponding to p19) was to study the reactivity of the recombinant proteins with the antiserum of individuals exposed to malaria, this reflecting the native proteins as "seen" by the human immune system.

Thus soluble recombinant PvMSP-1 p19 and PcMSP-1 p19 antigens purified by immunoaffinity were analysed by immunoblot using SDS-PAGE (15%) in the presence (reduced) or absence (non reduced) of DTT. Samples were charged onto gel after heating to 95°C in the presence of 2% SDS. The irreversible reduction was carried out as follows: the protein was resuspended in 0.2 M Tris-HCl, pH 8.4, 100 mM DTT, 1.0% SDS and heated for 30 minutes at 70°C. After diluting with water, acrylamide was added to a final concentration of 2 M and the mixture was incubated under nitrogen in the dark for 1 hour at 37°C. The immunoblot was revealed with a mixture of 25 human antisera originating from subjects with an acquired immunity to malaria due to *Plasmodium vivax*. V and C respectively

designate proteins derived from the MSP-1 of *P. vivax* and *P. cynomolgi*. It should be noted that irreversibly reduced recombinant proteins exhibited no reactivity with human antiserum while non irreversibly reduced proteins or non reduced proteins exhibited good reactivity. (The non reduced Pv MSP-1 p19 was a little weak since in its glycosylated state it does not bind well to nitro-cellulose paper). These results show that recognition of baculovirus MSP-1 p19 molecules by human antiserum is largely if not completely dependent on conformational epitopes sensitive to reduction which are reproduced in this system.

**Figure 3** - The soluble PvMSP1<sub>p42</sub> recombinant antigen (Longacre et al., 1994, op. Cit.) was incubated for 5 hours at 37°C in the presence of protein fractions derived from merozoites of *P. falciparum* and separated by isoelectrofocussing. The samples were then analysed by immunoblot in the presence (reduced) or absence (non reduced) of  $\beta$ -mercaptoethanol. Isoelectrofocussing fractions 5 to 12, and two total merozoite extracts made in the presence (Tex) or absence (T) of detergent, were analysed. The immunoblot was revealed with monoclonal antibodies specific for MSP1<sub>p42</sub> and p19 of *P. vivax*. The results suggest that there is a proteolytic activity in the *P. falciparum* merozoites which can be extracted with detergent. Digestion of p42 in certain fractions appear to cause polymerisation of the digestion products (p19); this polymerisation is probably linked to the formation of disulphide bridges since in the presence of  $\beta$ -mercaptoethanol, the high molecular weight forms disappear in favour of a molecule of about 19 kDa (Tex-R). The p19 polymerisation observed in these experiments could thus be an intrinsic property of this molecule *in vivo*.

**Figure 3B: The differential contribution of p42 and p19 antigens to the *P. vivax* anti-MSP-1 human response.**

Recognition of *P. vivax* MSP-1 p42 and p19 antigens by the antiserum of individuals with an acquired immunity to *P. vivax* was compared using the ELISA inhibition technique as follows: a mixture of 25 human antisera originating from subjects with an acquired immunity to

malaria due to *P. vivax* was diluted to 1:5000 and incubated for 4 hours at ambient temperature either alone, or in the presence of a 1 mM purified *P. vivax* recombinant p42 or p19. This mixture was transferred to a microtitre well which had been coated for 18 hours at 4°C with 500 ng.ml<sup>-1</sup> of purified absorbed recombinant p42 or p19, and incubated for 30 minutes at ambient temperature. After washing with PBS containing 0.1% of Tween 20, a goat anti-mouse IgG conjugated with peroxidase was added and the mixture was incubated for 1 hour at 37°C. The enzymatic activity was revealed by reading the optical density at 492 nm. The percentage inhibition was calculated based on values of 100% of antiserum activity with the coated antiserum on the microtitre plate in the absence of a competing antigen. Statistical data were calculated using a Statview program. Each bar represents the average percentage inhibition of a pair of competing/absorbed antigens based on 4 to 12 determinations; the vertical lines correspond to a 95% confidence interval. Asterisks (\*) designate the antigens produced in the presence of tunicamycin, thus with no N-glycosylation. The important parameters of these measurements were the dilution of the antiserum by 1:5000 which is in the region which is sensitive to ELISA curves and the competing antigen concentrations of 1 mM which includes competition by low affinity epitopes. Thus these data reflect the maximum resemblance between the two compared antigens. The results show that the majority, if not all of the p42 epitopes recognised by the human antiserum are present on the p19 since in the presence of the latter, the reactivity of the human antiserum against p42 is inhibited as much as by the p42 antigen itself. In contrast, however, about 20% of the p19 epitopes recognised by human antiserum were not or were not accessible on the p42, since the reactivity of the human antiserum against the p19 was much less inhibited by p42 than by p19 itself. Such specific epitopes of p19 can be constituted or revealed only after cleaving the p42 into p19 and p33. These results were not affected by glycosylation showing that the effect is really due to a difference between the peptide components of p19

and p42 and not to a difference in glycosylation. These results underline the fact that p19 has a distinct immunological identity to p42.

**Description of the PcMSP1<sub>p19</sub>S (soluble) construction (soluble p19 of *P. cynomolgi*)**

5 The DNA used for the above construction was obtained from a clone of the *Plasmodium cynomolgi* ceylonensis strain (22-23). This strain had been maintained by successive passages through its natural host (*Macaca sinica*) and cyclic transmissions via mosquitoes (27).

10 Blood parasites in the mature schizont stage were obtained from infected monkeys when the parasitemia had attained a level of 5%. They were then purified using the methods described in (25). The DNA was then extracted as described in (26).

A 1200 base pair fragment was produced using a PCR reaction using the oligonucleotides underlined in Figure 4 originating from *P. vivax*. The 5' oligonucleotide comprised an EcoRI restriction site and the 3' oligonucleotide comprised two synthetic TAA stop codons followed by a BglII restriction site. This fragment was introduced by ligation and via these EcoRI and BglII sites into the pVLSV<sub>200</sub> plasmid already containing the signal sequence for the MSP-1 protein of *P. vivax* (19). The new plasmid (pVLSV<sub>200</sub>C<sub>42</sub>) was used to analyse the DNA sequences.

20 The *P. cynomolgi* and the corresponding *P. vivax* sequences were aligned. The black arrows designate the presumed primary and secondary cleavage sites. They were determined by analogy with known sites in *P. falciparum* (27, 28). The vertical lines and horizontal arrows localise the limits of the four regions which were studied. Region 4 corresponded to the sequence coding for the *P. cynomolgi* p19. Glycosylation sites are boxed and the preserved cysteines are underlined. The lower portion of Figure 4 shows the percentage identity between the two isolates of *P. vivax* and *P. cynomolgi*.

30 The recombinant construction PcMSP1<sub>p19</sub>S contains the DNA corresponding to 8 base pairs of the leader sequence and the first 32

amino acids of the MSP-1 of *Plasmodium vivax* from Met<sub>1</sub> to Asp<sub>32</sub> (Belem isolate; Del Portillo et al., 1991, P. N. A. S., 88, 4030) followed by GluPhe, due to the EcoR1 site, connecting the two fragments. This is followed by the sequence coding for the *Plasmodium cynomolgi* MSP1<sub>p19</sub> from Lys<sub>276</sub> to Ser<sub>380</sub> (Ceylon strain). The construction was terminated by two TAA stop codons. This construction gave rise to a recombinant protein which was secreted in the culture supernatant of infected cells.

**Purification of recombinant PfMSP1p19 protein by immunoaffinity chromatography with a monoclonal antibody specifically recognising the p19 of *Plasmodium falciparum***

The chromatographic resin was prepared by binding 70 mg of a monoclonal antibody (obtained from a G17.12 hybridoma deposited at the CNCM [National Collection of Microorganism Cultures] (Paris, France) on the 14<sup>th</sup> February 1997, registration number I-1846; this G17.12 hybridoma was constructed from X63 Ag8 653 myeloma producing IgG 2a/k recognising the *P. falciparum* p19) to 3 g of activated CNBr-Sepharose 4B (Pharmacia) using standard methods detailed in the procedure employed by Pharmacia. The culture supernatants containing the soluble PfMSP1p19 were batch incubated with the chromatographic resin for 16 hours at 4°C. The column was washed once with 20 volumes of 0.05% NP40, 0.5 M of NaCl, PBS; once with 5 volumes of PBS and once with 2 volumes of 10 mM of sodium phosphate, pH 6.8. Elution was carried out with 30 ml of 0.2 M glycine, pH 2.2. The eluate was neutralised with 1 M sodium phosphate, pH 7.7 then concentrated by ultrafiltration and dialysed against PBS. To purify the anchored PfMSP1p19, all of the washing and elution solutions contained a supplemental 0.1% of 3-(dimethyl-dodecylammonio)propane sulphonate (Fluka).

**Recombinant *Plasmodium vivax* (p42 and p19) MSP1 vaccination test in the squirrel monkey *Saimiri sciureus***

This vaccination test was carried out on male non splenectomised 2 to 3 year old *Saimiri sciureus boliviensis* monkeys. Three monkeys were

injected 3 times intramuscularly at 3 week intervals with a mixture of about 50 to 100 µg each of recombinant soluble PvMSP1<sub>p42</sub> and p<sub>19</sub> (19), purified by immunoaffinity. Complete and incomplete Freund adjuvant was used as follows: 1<sup>st</sup> injection: 1:1 FCA/FIA; 2<sup>nd</sup> injection: 1:4 FCA/FIA; 3<sup>rd</sup> injection: 5 FIA. These adjuvant compositions were then mixed 1:1 with the antigen in PBS. Five control monkeys received the glutathione-S-transferase (GST) antigen produced in *E. coli* using the same protocol. The challenge infection was carried out by injecting  $2 \times 10^6$  red blood cells infected with an adapted *Plasmodium vivax* strain (Belem) 2.5 weeks after the final 10 injection. The protection was evaluated by determining parasitemia daily in all animals by examining smears stained with Giemsa.

The curves in Figure 5 show the variation in the measured parasitemia as the number of parasited red blood cells per microlitre of blood (up the ordinate, logarithmic scale) as a function of the time passed 15 after infection (in days). Curve A corresponds to the average values observed in the three vaccinated monkeys; curve B corresponds to the average values in the five control monkeys.

An examination of the Figure shows that the effect of the vaccination was to greatly reduce the parasitism.

#### 20 **Recombinant *Plasmodium cynomolgi* (p42 and p19) MSP1 vaccination test in the toque macaque *Macaca sinica***

Fifteen captured monkeys were used as follows: (1) 3 animals injected with 100 µg of soluble PcMSP1<sub>p42</sub>; (2) 3 animals injected with 35 µg (1<sup>st</sup> injection) or 50 µg (2<sup>nd</sup> and 3<sup>rd</sup> injections) of soluble PcMSP1<sub>p42</sub>; 25 (3) 3 animals injected with a mixture of PcMSP1<sub>p42</sub> and p<sub>19</sub>; (4) 3 animals injected with adjuvant plus PBS; (5) 3 animals not injected. Complete and incomplete Freund adjuvant was used in the protocol described above. Injections were intramuscular at 4 week intervals. The challenge infection was made by injecting  $2 \times 10^5$  red blood cells infected with *Plasmodium* 30 *cynomolgi* 4 weeks after the last injection. Protection was evaluated by determining parasitemia daily in all animals by examining the parasitemia



with Giemsa. Parasitemia were classified as negative only after counting 400 smear fields. The parasitemia were expressed as a percentage of parasitised red blood cells.

Figures 6A - 6G show the results obtained. Each of them shows parasitemia (expressed as the percentage of parasitised red blood cells along the ordinate on a logarithmic scale) observed in the challenge animals as a function of the time after infection (in days along the abscissa).

The results relate to:

- in Figure 6A; non vaccinated control animals;
- Figure 6B relates to animals which received a saline solution also containing Freund adjuvant;
- Figure 6C is a superposition of figures 6A and 6B, with the aim of highlighting the relative results resulting from administration of Freund adjuvant to the animals (the variations are clearly not significant);
- Figure 6D provides the results obtained at the end of vaccination with p42;
- Figure 6E concerns animals vaccinated with p19 alone;
- finally, Figure 6F concerns animals vaccinated with a mixture of p19 and p42.

The p42 certainly induced a certain level of protection. However, as shown in Figures 6E and 6F, the protection conferred by the recombinant p19 of the invention was considerably better.

The hypothesis can be formulated that the improved protection resulting from secondary cleavage of p42 which is accompanied by revealing free cysteine which, as a result, forms intermolecular bridges giving rise to p19 multimers which are highly characteristic of this form in recombinant proteins of the three species tested.

The numbers used to produce graphs (6A-6F) are given in Figure 6G.

***P. cynomolgi* toque macaque vaccination test; second challenge  
infection of monkeys vaccinated with p19 alone and controls (Figures  
8)**

Six months later, with no other vaccination, the 3 macaques which  
5 received the p19 MSP-1 alone with FCA/FIA (Figure 6E) and the 3  
macaques which received a saline solution containing Freund adjuvant  
(Figure 6B) and 2 new unaffected unvaccinated monkeys underwent a new  
challenge infection by injecting  $1 \times 10^6$  red blood cells infected with  
*Plasmodium cynomolgi*. Protection was evaluated by determining  
10 parasitemia daily in all animals by examining Giemsa smears. The  
parasitemia were classified negative only after counting 400 smear fields.  
The parasitemia were expressed as the percentage of parasitised red  
blood cells (the figures used to produce graphs 8A-C are given in Figure  
8D). The six immunised animals which underwent challenge infection six  
15 months earlier had no detectable parasitemia except for 1 animal in each  
group which exhibited a parasitemia of 0.008% for 1 day (Figures 8A and  
8B). The two unaffected controls exhibited a conventional parasitemia with  
a maximum of 0.8% and for 21 days (Figure 8C). Thus the 3 animals  
vaccinated with the MSP-1 p19 were also protected six months later than  
20 the 3 controls which exhibited a complete conventional infection after the  
first challenge infection, despite the absence of or a very slight parasitemia  
after the first challenge infection. These results suggest that the protection  
period for p19 is at least six months.

**Vaccination test with p19 in association with alum in the *P. cynomolgi*  
25 toque macaque system (Figures 9)**

The previous positive protection results were obtained using  
complete (FCA) or incomplete (FIA) Freund adjuvant. However, the only  
adjuvant which is currently allowed in man is alum. For this reason, we  
carried out a vaccination test with *P. cynomolgi* MSP-1 p19 in the toque  
30 macaque in the presence of alum as the adjuvant. Six captured macaques  
were used as follows: (1) 3 animals injected with 4 doses of 50 mg of

recombinant *P. cynomolgi* MSP-1 p19 with 20 mg of alum; (2) 3 animals injected 4 times with physiological water and 10 mg of alum. The injections were intramuscular at 4 week intervals. The challenge infection was made by injecting  $2 \times 10^5$  red blood cells infected with *P. cynomolgi* 4 weeks after the last injection. Protection was evaluated by daily determination of parasitemia in all animals by examining Giemsa smears. The parasitemia were classified negative only after counting 400 smear fields. Parasitemia were expressed as the percentage of parasitised red blood cells. The results of this experiment were as follows. 2 of the 3 macaques immunised with recombinant p19 with alum had about 30 times less total parasitemia during the infection period (Figures 9A and 9B) than the 3 control macaques immunised with physiological water and alum (Figure 9D) after the challenge infection. The third macaque immunised with p19 (Figure 9C) was not very different from the controls. For the vaccination test using *Plasmodium cynomolgi* p19 in the toque macaque, *macaca sinica*, described in Figure 9, the data used to produce the graphs (9A-9D) are given in (Figure 9E). While the results are a little less spectacular than the preceding results (Figures 6, 8), this is the first time that significant protection has been observed for recombinant MSP-1 with alum.

**Figure 10: Vaccination test with a recombinant *Plasmodium falciparum* p19 in the squirrel monkey**

Twenty *Saimiri sciureus guyanensis* (squirrel monkeys) of about 3 years old raised in captivity were used as follows: (1) 4 animals injected with 50 mg of soluble Pf MSP-1 p19 in the presence of Freund adjuvant as follows: 1<sup>st</sup> injection: 1:1 FCA/FIA; 2<sup>nd</sup> injection: 1:4 FCA/FIA; 3<sup>rd</sup> injection: FIA. These adjuvant compositions were then mixed with 1:1 antigen in PBS; (2) 2 control animals received Freund adjuvant as described for (1) with only PBS; (3) 4 animals injected with 50 mg of soluble Pf MSP-1 p19 in the presence of 10 mg of alum (Alu-Gel-S, Serva); (4) 2 control animals received 10 mg of alum with only PBS; (5) 4 animals injected with about 50-100 mg of GPI anchored Pf MSP-1 p19 reconstituted into liposomes as

follows: 300 mmoles of cholesterol and 300 mmoles of phosphatidyl choline were vacuum dried and resuspended in 330 mM of N-octylglucoside in PBS with 1.4 mg of Pf MSP-1 p19, GPI. This solution had been dialysed against PBS with adsorbent Bio-Beads SM-2 (Bio-Rad) and the liposomes formed were concentrated by centrifuging and resuspended in PBS. The 1<sup>st</sup> injection was made with fresh liposomes kept at 4°C and the 2<sup>nd</sup> and 3<sup>rd</sup> injections were made with liposomes which had been frozen for preservation; (6) 2 animals injected with control liposomes made in the same way, in the absence of the p19, GPI antigen as described for (5); (7) 2 animals injected with physiological water. Three intramuscular injections were made at 4 week intervals. The challenge infection was made by injecting  $1 \times 10^6$  red blood cells infected with *Plasmodium falciparum*. Protection was evaluated by determining parasitemia daily in all animals by examining the Giemsa smears. Parasitemia were expressed as the percentage of parasitised red blood cells. The results of this vaccination test are shown in Figures 10, A-G.

The groups immunised with p19 in Freund adjuvant or liposome demonstrated similar parasitemia to the control groups after a challenge infection (one animal (number 29) vaccinated with p19 in Freund adjuvant died several days after challenge infection for reasons independent of vaccination (cardiac arrest"). Irregularities in administration of the antigen in these 2 groups (poor Freund emulsion, congealed liposomes) did not allow the significance of these results to be completely evaluated. In the alum group, 2 animals showed total parasitemia for the duration of the infection about 4 times less than the controls, 1 animal about 3 times less and 1 animal was similar to the controls. This experiment was a little difficult to interpret due to the variability in the controls, probably due to the strain of parasite used for the challenge infection which would not have been quite adapted to the non splenectomised *Saimiri* model developed only recently in Cayenne. However, the real effect with alum, although imperfect, is encouraging in that our antigens seem to be the only recombinant P.

*falciparum* MSP-1 versions which currently have shown a certain effectiveness in combination with alum.

**Vaccination test with a recombinant *Plasmodium falciparum* p19 in the squirrel monkey (same test as for Figures 10)**

Monkeys bred in captivity were injected intramuscularly with 1 ml of inoculum twice at 4 week intervals as follows: (1) 4 animals injected with 50 µg of soluble PfMSP1p19 in the presence of Freund adjuvant as follows: 1<sup>st</sup> injection: 1:1 FCA/FIA; 2<sup>nd</sup> injection: 1:4 FCA/FIA; and mixed then 1:1 with the antigen in PBS; (2) 4 animals injected with 50 µg of soluble PfMSPp19 in the presence of 10 mg of alum; (3) 4 animals injected with about 50 µg of GPI anchored PfMSP1p19 reconstituted into liposomes composed of 1:1 molar cholesterol and phosphatidyl choline. The animals were bled 17 days after the second injection.

Red cells from a squirrel monkey with 30% parasitemia due to *P. falciparum* (with the mature forms in the majority) were washed with PBS and the residue was diluted 8 times in the presence of 2% SDS and 2% dithiothreitol and heated to 95° before being charged onto a polyacrylamide gel of 7.5% (separation gel) and 4% (stacking gel). After transfer to nitrocellulose, immunoblot analysis was carried out with antisera as follows: (1) pool of antisera of 4 monkeys vaccinated with soluble PfMSP1p19 in Freund adjuvant, twentieth dilution; (2) pool of antisera of 4 monkeys vaccinated with soluble PfMSP1p19 in alum adjuvant, twentieth dilution; (3) pool of antisera of 4 monkeys vaccinated with anchored PfMSP1p19 in liposomes, twentieth dilution; (4) monoclonal antibody, which reacts with a linear epitope of PfMSP1p19, 50 mg/ml; (5) SHI90 antisera pool originating from about twenty monkeys repeatedly infected with *P. falciparum* and which had become unaffected by any subsequent infection with *P. falciparum*, five hundredth dilution; (6) antiserum pool of unaffected monkeys (never exposed to *P. falciparum*), twentieth dilution.

The results show that the 3 antiserum pools of monkeys vaccinated with PfMSP1p19 reacted strongly and specifically with very high molecular

weight complexes (diffuse in the stacking gel) and present in parasite extracts containing more mature forms. These results support the hypothesis that a specific aggregate of PfMSP1p19 is present *in vivo* comprising epitopes which are reproduced in recombinant PfMSP1p19 molecules synthesised in the baculovirus system, in particular oligomeric forms thereof.

Figure 7 also illustrated these results. It shows immunoblots produced on gel. The first three gel tracks illustrate the *in vivo* response of monkeys to injections of p19 [(1) with Freund adjuvant, (2) with alum, (3) in the form of a liposome] and in particular the existence of high molecular weight complexes supporting the hypothesis of *in vivo* aggregation of p19 in the form of an oligomer, specific to the maturation stage (when p42 is cut into p19 and p33).

This vaccination test also comprises a third injection identical to the previous injections. The injection with Freund adjuvant contained only FIA.

There were two animal controls for each group, namely: 2 control animals injected with PBS and Freund adjuvant; 2 control animals injected with PBS and alum; 2 control animals injected with liposomes without protein; and two control animals injected with PBS without adjuvant.

Protection was evaluated as described above.

Figure 7B: The data for this Figure were derived from the squirrel monkey *P. falciparum* / vaccination test (Figure 10 below). The numbers correspond to the individual monkeys noted in Figure 10. The techniques and methods for this Figure were the same as for Figure 7 except that the individual antiserum for each monkey was tested after three injections the day of the proof injection and the SHI antiserum was diluted by 1:250. The results show that the antiserum for 4 monkeys vaccinated with p19 and alum reacted strongly and specifically with very high molecular weight complexes while the monkeys of other groups vaccinated with p19 and Freund adjuvant or liposomes showed only a little reactivity with these complexes. Since the monkeys vaccinated with p19 and alum were also

the best protected, this reactivity with the high molecular weight complexes appeared to indicate a protective effect, despite one monkey in the group not being protected with respect to the controls and that another was only partially protected.

- 5       The invention also, of course, concerns other applications, for example those described below with respect to certain of the examples, although these are not limiting in character.

#### Therapy

- 10       The recombinant molecule PfMSP1p19 can be used to produce specific antibodies which can possibly be used by passive transfer for therapeutics for severe malaria due to *P. falciparum* when there is a risk of death.

#### Diagnostics

- 15       The recombinant molecules PvMSP1p42, PvMSP1p19 and PfMSPp19 derived from baculovirus can and have been used to produce specific murine monoclonal antibodies. These antibodies, in combination with polyclonal anti-MSP1p19 antisera originating from another species such as the rabbit or goat can form the basis of a semi-quantitative diagnostic test for malaria which can distinguish between malaria due to *P.*  
20       *falciparum*, which can be fatal, and malaria due to *P. vivax*, which is generally not fatal. The principle of this test is to trap and quantify any MSP-1 molecule containing the p19 portion in the blood.

In this context, the advantages of the MSP1p19 molecule are as follows:

- 25       (i) It is both extremely well conserved in the same species and sufficiently divergent between different species to enable specific species reactants to be produced. No cross reaction has been observed between antibodies derived from PfMSP1p19 and PvMSP1p19;  
30       (ii) the function of MSP1p19, while not known with precision, seems to be sufficiently important that this molecule does not vary significantly or is deleted without lethal effect for the parasite;

(iii) it is a major antigen found in all merozoites and thus it must in principle be detectable even at low parasitemia and proportionally to the parasitemia;

(iv) since the recombinant MSP1p19 molecules derived from baculovirus appear to reproduce more of the native structure of MSP1p19, the antibodies produced against these proteins will be well adapted to diagnostic use.

The microorganisms identified below have been deposited under Rule 6.1 of the Treaty of Budapest of 1<sup>st</sup> February 1996, under the following registration numbers:

<u>Identification reference</u>	<u>Registration numbers</u>
PvMSP1p19A	I-1659
PvMSP1p19S	I-1660
PfMSP1p19A	I-1661
PfMSP1p19S	I-1662
PcMSP1p19S	I-1663

The invention also concerns the use of these antibodies, preferably fixed to a solid support (for example for affinity chromatography) for the purification of type p19 peptides initially contained in a mixture.

Purification means bringing this mixture into contact with an antibody, dissociating the antigen-antibody complex and recovering the purified p19 type peptide.

The invention also concerns vaccine compositions, also comprising mixtures of proteins or fragment, in particular mixtures of the type:

- *P. falciparum* p19 and *P. vivax* p19;
- *P. falciparum* p19 and *P. falciparum* p42, the latter if necessary being deprived of its most hypervariable regions;



- *P. vivax* p19 and *P. vivax* p42, the latter if necessary being deprived of its most hypervariable regions;
- *P. falciparum* p19 and *P. falciparum* p42, the latter if necessary being deprived of its most hypervariable regions, and *P. vivax* p19 and *P. vivax* p42, the latter if necessary being deprived of its most hypervariable regions.

In the present case, the most hypervariable regions are defined as regions I or region II and all or part of region III, the portion of region III which is preferably deleted being that which is juxtaposed to region II (the conserved portion being located to the side of the C-terminal of p33, close to the p19). Regions II and III are illustrated in Figure 4.

The invention is not limited to the production of human vaccine. It is also applicable to the production of veterinary vaccine compositions using the corresponding proteins or antigens derived from parasites which are infectious for mammals and products under the same conditions. It is known that infections of the same type, babesiosis, also appear in cattle, dogs and horses. One of the antigens of the *Babesia* species has a high conformational homology (in particular in the two EFG-like and cysteine-rich domains) and functional homology with a protein portion of MSP-1 [(36), (37) and (38)].

Examples of veterinary vaccines using a soluble antigen against such parasites have been described (39).

It goes without saying that the p19s used in these mixtures can also be modified as described in the foregoing when considered in isolation.

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The invention also concerns hybridomas secreting specific antibodies selectively recognising the p19 of a MSP-1 protein in the merozoite form of a *Plasmodium* type parasite which is infectious for man other than *Plasmodium vivax* and which does not recognise *Plasmodium vivax*.

In particular, these hybridomas secrete monoclonal antibodies which do not recognise *Plasmodium vivax* and which specifically recognise *Plasmodium falciparum* p19.

The invention also concerns a hybridoma characterized in that it produces a specific antibody which specifically recognises the p19 of *P. vivax* and the p19 of *P. cynomolgi*. A F10-3 hybridoma has been constructed from the X63 Ag8 653 myeloma producing IgG 2b/k recognising the p42 glycoprotein of *Plasmodium vivax*.

## CLAIMS

1. A recombinant protein whose essential constituent polypeptide sequence is:
- either that of a 19 kilodalton (p19) C-terminal fragment of the surface protein 1 of the merozoite form (MSP-1 protein) of a *Plasmodium* type parasite other than *Plasmodium vivax* which is infectious for mammals, in particular for man, the C-terminal fragment remaining normally anchored to the parasite surface at the end of its penetration phase into human erythrocytes in the event of an infectious cycle;
  - or that of a portion of that fragment which is also capable of inducing an immune response which can inhibit *in vivo* parasitemia due to the corresponding parasite;
  - or that of a peptide which is capable of inducing a cellular and/or humoral immunological response equivalent to that produced by said p19 fragment or said portion of that fragment; and
- said recombinant protein possibly further comprising conformational epitopes which are unstable in a reducing medium and which constitute the majority of the epitopes recognised by human antisera formed against the corresponding *Plasmodium*.
2. A recombinant protein according to claim 1, characterized in that it is recognised by human antisera formed against the corresponding *Plasmodium* or against a homologous *Plasmodium* when it is in its non reduced state or in a reduced non irreversible state, but is not recognised or is only recognised to a slight extent by these same antisera when it is irreversibly reduced.
3. A protein according to claim 1 or claim 2, characterized in that it inhibits the reactivity of an immune human antiserum with the corresponding p42 produced under the same conditions, also the

p42 itself and in that the p42 can only partially inhibit the reactivity of said human antiserum against the p19.

4. A recombinant protein according to any one of claims 1 to 3, characterized in that it is essentially deprived of any polypeptide sequence normally upstream of the C-terminal polypeptide sequence of the p33 (33 kDa N-terminal fragment) normally on the side associated with the p19 in the corresponding p42, before natural cleavage of the latter, said last C-terminal polypeptide sequence of the p33 containing less than 50 amino acid residues when it is present.

5. A recombinant protein according to any one of claims 1 to 3, characterized in that it is essentially deprived of any polypeptide sequence normally upstream of the C-terminal polypeptide sequence of the p33 (33 kDa N-terminal fragment) normally associated with the p19 in the corresponding p42 before natural cleavage of the latter, said last C-terminal polypeptide sequence of the p33 containing less than 10 amino acid residues when it is present.

6. A recombinant protein according to any one of claims 1 to 3, characterized in that it is essentially deprived of any polypeptide sequence normally upstream of the C-terminal polypeptide sequence of the p33 (33 kDa N-terminal fragment) normally associated with the p19 in the corresponding p42 before natural cleavage of the latter, said last C-terminal polypeptide sequence of the p33 being limited, when it is present, to that which retains a substantial degree of conservation in *Plasmodium* which are infectious for man, such as *P. falciparum* or *P. vivax*.

7. A recombinant protein according to any one of claims 1 to 6, characterized in that the portion of p19 fragment contains at least one of the two EGF regions normally contained in this p19.



8. A recombinant protein according to any one of claims 1 to 7, characterized in that the molecular weight of said p19 fragment or of said portion of p19 fragment is in the range 10 to 25 kDa, in particular in the range 10 to 15 kDa.
- 5 9. A recombinant protein according to any one of claims 1 to 8, characterized in that it also comprises a glycosylphosphatidylinositol (GPI) group of the type enabling the p19 fragment to anchor to the host cell, in particular a eukaryote cell, preferably a cell of an insect infectable by a baculovirus, in which said recombinant protein is expressed.
- 10 10. A recombinant protein according to any one of claims 1 to 8, characterized in that it is deprived of the extremely hydrophobic C-terminal portion which intervenes in induction of anchoring of said recombinant protein to the cell membrane of the host in which it is expressed, in particular in a eukaryote cell, preferably a cell of an insect infectable by a baculovirus.
- 15 11. A recombinant protein according to claim 10, characterized in that it is hydrosoluble.
12. A recombinant protein according to any one of claims 1 to 11, characterized in that it contains the p19 sequence of the MSP-1 protein of *Plasmodium falciparum* or said portion of the corresponding fragment.
- 20 13. A recombinant protein according to any one of claims 1 to 12, characterized in that it contains the p19 sequence of the MSP-1 protein of *Plasmodium cynomolgi* or said portion of the corresponding fragment.
- 25 14. An oligomer of the recombinant protein according to any one of claims 1 to 13.
15. An oligomer according to claim 14, characterized in that it contains 2 to 50 monomer units of said polypeptide sequence of the recombinant protein as defined in any one of claims 1 to 13.
- 30

16. A recombinant protein according to any one of claims 1 to 14, characterized in that it is conjugated to a carrier molecule for use in the production of vaccines.
17. A vaccination composition against a *Plasmodium* type parasite which is infectious for man, containing as an active principle a recombinant protein whose essential constituent polypeptide sequence is:
- either that of a 19 kilodalton (p19) C-terminal fragment of the surface protein 1 of the merozoite form (MSP-1 protein) of a *Plasmodium* type parasite which is infectious for man, said C-terminal fragment remaining normally anchored to the parasite surface at the end of its penetration phase into human erythrocytes in the event of an infectious cycle;
  - or that of a portion of that fragment which is also capable of inducing an immune response which can inhibit *in vivo* parasitemia due to the corresponding parasite;
  - or that of a peptide which is capable of inducing a cellular and/or humoral immunological response equivalent to that produced by said p19 fragment or said portion of that fragment; and
- said recombinant protein further comprising conformational epitopes which are unstable in a reducing medium and which constitute the majority of the epitopes recognised by human antisera formed against the corresponding *Plasmodium*.
18. A vaccinating composition according to claim 17, characterized in that its active principle consists of a recombinant protein according to any one of claims 2 to 13 or 16, or an oligomer according to claim 14 or claim 15.
19. A vaccinating composition against a *Plasmodium* type parasite which is infectious for man, containing as an active principle an oligomer of a recombinant protein according to claim 17 or claim 18.

20. An antibody specifically recognising the p19 of a MSP-1 protein of the merozoite form of a *Plasmodium* type parasite which is infectious for man other than *Plasmodium vivax* and which does not recognise *Plasmodium vivax*.
- 5 21. An antibody according to claim 20, characterized in that it is monoclonal.
22. An antibody according to claim 20, characterized in that it is monoclonal and in that it specifically recognises the p19 of *P. falciparum*.
- 10 23. A monoclonal antibody according to claim 20, characterized in that it specifically recognises the p19 of *P. vivax*.
24. A differential diagnostic process to distinguish between a parasitic infection due to *P. vivax* and a parasitic infection due to another *Plasmodium*, characterized by bringing a biological sample infected with *Plasmodium* into contact with an antibody according to claim 23 and with an antibody according to claim 21 or claim 22, and detecting the production or non-production of an immunological reaction depending on the case.
- 15 25. A recombinant baculovirus type modified vector containing, under the control of a promoter contained in the vector and able to be recognised by cells transfectable by said vector, a first nucleotide sequence coding for a signal peptide which is compatible with expression in a baculovirus system, characterized by a second sequence downstream of the first, also under the control of said promoter and coding for the peptide sequence:
- 20
- 25
- either of a 19 kilodalton (p19) C-terminal fragment of the surface protein 1 of the merozoite form (MSP-1 protein) of a *Plasmodium* type parasite which is infectious for man, the C-terminal fragment remaining normally anchored to the parasite surface at the end of

its penetration phase into human erythrocytes in the event of an infectious cycle;

- or of a portion of that peptide fragment provided that the expression product from the second sequence in a baculovirus system is also capable of inducing an immune response which can inhibit *in vivo* parasitemia due to the corresponding parasite;
- or of a peptide which is capable of inducing a cellular and/or humoral immunological response equivalent to that produced by said peptide fragment p19 or said peptide fragment portion; and

said nucleotide sequence also having a G and C content in the range 40% to 60%, preferably at least 50%, of the totality of nucleotides from which it is constituted.

26. A modified vector according to claim 25, characterized in that the said second polypeptide sequence is in accordance with that defined in any one of claims 2 to 13.

27. A modified vector according to claim 25, characterized in that the second nucleotide sequence is a synthetic sequence.

28. A modified vector according to any one of claims 25 to 27, characterized in that the first nucleotide sequence codes for a signal peptide from *Plasmodium vivax* and normally associated with the *Plasmodium* MSP-1 protein.

29. A modified vector according to any one of claims 25 to 28, characterized in that the second nucleotide sequence is deprived at its 3' terminal end of the hydrophobic C-terminal end sequence which is implicated in induction of anchoring said recombinant protein to the cell membrane of the host in which it is expressed, in particular in a cell of an insect infectable by a baculovirus.

30. A modified vector according to any one of claims 25 to 29, characterized in that it consists of a modified baculovirus.

31. An organism, in particular an Sf9 type insect cell, transfectable and transfected by the modified vector according to any one of claims 25 to 29.
32. A synthetic DNA containing a first nucleotide sequence for which at least a portion codes for the peptide sequence:
- either of a 19 kilodalton (p19) C-terminal fragment of the surface protein 1 of the merozoite form (MSP-1 protein) of *Plasmodium falciparum*, said C-terminal fragment remaining normally anchored to the parasite surface at the end of its penetration phase into human erythrocytes in the event of an infectious cycle;
  - or of a portion of that peptide fragment provided that the expression product of said DNA in a baculovirus system is also capable of inducing an immune response which can inhibit *in vivo* parasitemia due to the corresponding parasite;
  - or a peptide capable of inducing a cellular and/or humoral type immunological response equivalent to that produced by said p19 peptide fragment or said portion of that fragment; and
- said nucleotide sequence also having a G and C nucleotide content in the range 40% to 60%, preferably at least 50%, of the totality of nucleotides from which said synthetic DNA is constituted.
33. A synthetic DNA sequence according to claim 32, characterized in that its first nucleotide sequence is deprived at its 3' terminal end of the sequence coding for the hydrophobic C-terminal end region normally implicated in inducing anchoring of the p19 protein to the cell membrane of the host in which it is expressed, in particular in a cell of an insect infectable by a baculovirus.
34. A synthetic DNA sequence according to claim 32 or claim 33, characterized in that the first nucleotide sequence is preceded by a signal nucleotide sequence coding for a signal peptide normally

associated with a *Plasmodium* MSP-1 protein, homologous or heterologous relative to the principal sequence.

35. A synthetic DNA sequence according to claim 34, characterized in that the signal sequence originates from *P. vivax*.

5 36. A synthetic DNA according to any one of claims 32 to 35, characterized in that said first nucleotide sequence includes a 3'-terminal sequence coding for a polypeptide cell membrane anchoring region, said anchoring region fixing the expressed recombinant protein to the surface of the membrane of the host cell transformed with a vector containing said synthetic DNA, said 3' sequence being homologous to that of the principal nucleotide sequence, or heterologous, in particular that from *P. vivax*.

37. A synthetic DNA according to claim 36, characterized in that the 3'-terminal sequence originates from *P. vivax*.

15 38. A synthetic DNA sequence according to any one of claims 32 to 36, characterized in that it is deprived of said 3'-terminal sequence.

39. A baculovirus type vector according to claim 25, characterized in that it is selected from:

- the virus deposited at the CNCM [Collection Nationale de Cultures de Microorganismes; National Collection of Microorganism Cultures] with registration number I-1659;
- the virus deposited at the CNCM with registration number I-1660;
- the virus deposited at the CNCM with registration number I-1661;
- the virus deposited at the CNCM with registration number I-1662;
- 25 • the virus deposited at the CNCM with registration number I-1663.

40. A hybridoma secreting monoclonal antibodies having the specifications of the antibodies of any one of claims 21 to 23.

41. A process for separating a p19 peptide with a given specificity from a mixture of peptides, characterized by bringing said peptide mixture into contact with a corresponding antibody, in accordance with any

30

one of claims 20 to 23, preferably already fixed on an insoluble support, by subsequently dissociating the antigen-antibody compound formed and by recovering the purified p19 peptide.

42. Use of a protein according to any one of claims 1 to 13 or of the oligomer according to claim 15 or 16 to prepare an immunogen composition which can induce an immune response against a *Plasmodium* infection.

43. A vaccine composition comprising, as active principles, a mixture of a protein according to any one of claims 1 to 13 and either the corresponding p42, with if necessary the least conserved portions deleted, or another recombinant p19 or p42 type protein, originating from a parasite homologous with that from which said protein originates.

44. A vaccine composition according to claim 23, characterized in that the mixture of active principles is selected from the following mixtures:

- *P. falciparum* p19 and *P. vivax* p19;
- *P. falciparum* p19 and *P. falciparum* p42;
- *P. vivax* p19 and *P. vivax* p42, if necessary deprived of its most hypervariable regions;
- *P. falciparum* p19 and *P. falciparum* p42, if necessary deprived of its most hypervariable regions and *P. vivax* p19 and *P. vivax* p42, if necessary deprived of its most hypervariable regions.

45. A hybridoma according to claim 40, characterized in that it has been deposited at the CNCM, (Paris, France) with registration number I-1846, on the 14<sup>th</sup> February 1997.

ABSTRACT

The invention relates to a recombinant protein fabricated in a baculovirus system, of which the essential constitutive polypeptide sequence is that of a C-terminal fragment of 19 kilodalton (p19) of the surface protein 1 (protein MSP-1) of the merozoite parasite of the *Plasmodium* type, particularly *Plasmodium falciparum*, which is infectious for humans, said C-terminal fragment remaining normally anchored at the surface of the parasite at the end of its penetration phase into human erythrocytes, in the occurrence of an infectious cycle. Said recombinant protein is applicable to the production of vaccines against malaria.



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FIGURE 1A

Bac 19    E F N I S Q H Q C V K K Q C P E N  
GAA TTC AAC ATC TCG CAG CAC CAA TGC GTG AAA AAA CCA TGT CCC GAG AAC  
PF 19    AAC ATT TCA CAA CAC CAA TGC GTA AAA AAA CCA TGT CCA GAA AAT

Bac 19    S G C F R H L D E R E E C K C L L  
TGT GGC TGT TTC AGA CAC TTG GAC GAG AGA GAG GAG TGT AAA TGT CTG CTG  
PF 19    TGT GGA TGT TTC AGA CAT TTA GAT GAA AGA GAA GAA TGT AAA TGT TTA TTA

Bac 19    N Y K Q E G D K C V E N P N P T C  
AAC TAC AAA CAG GAG GGC GAC AAG TGC GTG GAG AAC CCC AAC CCG ACC TGT  
PF 19    AAT TAC AAA CAA GAA GGT GAT AAA TGT GTT GAA AAT CCA AAT CCT ACT TGT

Bac 19    N E N N G G C D A D A K C T E E D  
AAC GAG AAC AAC GGC GGC TGT GAC CCA GAC GCC AAA TGC ACC GAG GAG GAC  
PF 19    AAC GAA AAT AAT GGT CCA TGT GAT CCA GAT GCC AAA TGT ACC GAA GAA GAT

Bac 19    S G S N G K K I T C E C T K P D S  
TCG GGC AGC AAC GGC AAG AAA ATC ACG TGT GAG TGT ACC AAA CCC GAC TCG  
PF 19    TCA GGT AGC AAC GGA AAG AAA ATC ACA TGT GAA TGT ACT AAA CCT GAT TGT

Bac 19    Y P L F D G I F C S \* \*  
TAC CCG CTG TTC GAC GGC ATC TTC TGC AGC TAA TAA  
PF 19    TAT CCA CTT TTC GAT GGT ATT TTC TGC AGT

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FIGURE 1B

	E	F	N	I	S	Q	H	Q	C	V	K	K	Q	C	P	E	N
Bac 19	GAA	TTT	AAC	ATC	TCG	CAG	CAC	CAA	TGC	GTG	AAA	AAA	CAA	TGT	CCC	GAG	AAC
PF 19			AAC	ATT	TCA	CAA	CAC	CAA	TGC	GTA	AAA	AAA	CAA	TGT	CCA	GAA	AAT
	S	G	C	F	R	H	L	D	E	R	E	E	C	K	C	L	L
Bac 19	TCT	GGC	TGT	TTC	AGA	CAC	TTG	GAC	GAG	AGA	GAG	GAG	TGT	AAA	TGT	CTG	CTG
PF 19	TCT	GGA	TGT	TTC	AGA	CAT	TTA	GAT	GAA	AGA	GAA	GAA	TGT	AAA	TGT	TTA	TTA
	N	Y	K	Q	E	G	D	K	C	V	E	N	P	N	P	T	C
Bac 19	AAC	TAC	AAA	CAG	GAG	GGC	GAC	AAG	TGC	GTG	GAG	AAC	CCC	AAC	CCG	ACC	TGT
PF 19	AAT	TAC	AAA	CAA	GAA	GGT	GAT	AAA	TGT	GTT	GAA	AAT	CCA	AAT	CCT	ACT	TGT
	N	E	N	N	G	G	C	D	A	D	A	K	C	T	E	E	D
Bac 19	AAC	GAG	AAC	AAC	GGC	GGC	TGT	GAC	GCA	GAC	GGC	AAA	TGC	ACC	GAG	GAG	GAC
PF 19	AAC	GAA	AAT	AAT	GGT	GGA	TGT	GAT	GCA	GAT	GGC	AAA	TGT	ACC	GAA	GAA	GAT
	S	G	S	N	G	K	K	I	T	C	E	C	T	K	P	D	S
Bac 19	TCG	GGC	AGC	AAC	GGC	AAG	AAA	ATC	ACG	TGT	GAG	TGT	ACC	AAA	CCC	GAC	TGG
PF 19	TCA	GCT	AGC	AAC	GGA	AAG	AAA	ATC	ACA	TGT	GAA	TGT	ACT	AAA	CCT	GAT	TCT
	Y	P	L	F	D	G	I	F	C	S	S	S	N	F	L	G	I
Bac 19	TAC	CCG	CTG	TTC	GAC	GGC	ATC	TTC	TGC	AGC	TCC	TCT	AAC	TTC	TTG	GGC	ATC
PF 19	TAT	CCA	CTT	TTC	GAT	GGT	ATT	TTC	TGC	AGT	TCC	TCT	AAC	TTC	TTA	GGA	ATA
	S	F	L	L	I	L	M	L	I	L	Y	S	F	I	*	*	
Bac 19	TCG	TTC	TTG	TTG	ATC	CTC	ATG	TTG	ATC	TTG	TAC	AGC	TTC	ATT	TAA	TAA	
PF 19	TCA	TTC	TTA	TTA	ATA	CTC	ATG	TTA	ATA	TTA	TAC	AGT	TTC	ATT			

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ATG	AAG	GCG	CTA	CTC	TTT	TTG	TTC	TCT	TTC	ATT	TTT	TTC	GTT	ACC	AAA	TGT
M	K	A	L	L	F	L	F	S	F	I	F	F	V	T	K	C
CAA	TGT	GAA	ACA	GAA	AGT	TAT	AAG	CAG	CTT	GTA	GCC	AAC	GTG	GAC	GAA	TTC
Q	C	E	T	E	S	Y	K	Q	L	V	A	N	V	D	<u>E</u>	<u>E</u>
AAC	ATC	TCG	CAG	CAC	CAA	TGC	GTG	AAA	AAA	CAA	TGT	CCC	GAG	AAC	TCT	GGC
N	I	S	Q	H	Q	C	V	K	K	Q	C	P	E	N	S	G
TGT	TTC	AGA	CAC	TTG	GAC	GAG	AGA	GAG	GAG	TGT	AAA	TGT	CTG	CTG	AAC	TAC
C	F	R	H	L	D	E	R	E	E	C	K	C	L	L	N	Y
AAA	CAG	GAG	GGC	GAC	AAG	TGC	GTG	GAG	AAC	CCC	AAC	CCG	ACC	TGT	AAC	GAG
K	Q	E	G	D	K	C	V	E	N	P	N	P	T	C	N	E
AAC	AAC	CCC	GGC	TGT	GAC	GCA	GAC	GCC	AAA	TGC	ACC	GAG	GAG	GAC	TCG	GGC
N	N	G	G	C	D	A	D	A	K	C	T	E	E	D	S	G
AGC	AAC	GGC	AAG	AAA	ATC	ACG	TGT	GAG	TGT	ACC	AAA	CCC	GAC	TCG	TAC	CCG
S	N	G	K	K	I	T	C	E	C	T	K	P	D	S	Y	P
CTG	TTC	GAC	GGC	ATC	TTC	TGC	AGC	TAA	TAA							
L	F	D	G	I	F	C	S	*	*							

FIGURE 1C

Year	Age	Sex	Height (cm)	Weight (kg)	Body mass index (kg/m <sup>2</sup> )	Waist circumference (cm)	Hip circumference (cm)	Waist-hip ratio	Visceral fat area (cm <sup>2</sup> )	Subcutaneous fat area (cm <sup>2</sup> )	Total fat area (cm <sup>2</sup> )	Visceral fat volume (L)	Subcutaneous fat volume (L)	Total fat volume (L)
1990	20	M	170	65	22.0	85	95	0.89	150	100	250	1.8	1.0	2.8
1995	25	F	160	55	21.5	75	85	0.88	120	80	200	1.5	0.8	2.3
2000	30	M	175	75	24.5	90	100	0.90	180	120	300	2.2	1.2	3.4
2005	35	F	165	60	21.2	80	90	0.89	140	90	230	1.7	0.9	2.6
2010	40	M	180	85	27.8	100	110	0.91	200	140	340	2.5	1.4	3.9
2015	45	F	170	70	24.2	95	105	0.90	190	130	320	2.4	1.3	3.7

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GAA	ACA	GAA	AGT	TAT	AAG	CAG	CTT	GTA	GCC	AAC	GTG	GAC	GAA	TTC		
E	T	E	S	Y	K	Q	L	V	A	N	V	D	E	F		
AAC	ATC	TCG	CAG	CAC	CAA	TGC	GTG	AAA	AAA	CAA	TGT	CCC	GAG	AAC	TCT	GGC
N	I	S	Q	H	Q	C	V	K	K	Q	C	P	E	N	S	G
TGT	TTC	AGA	CAC	TTG	GAC	GAG	AGA	GAG	GAG	TGT	AAA	TGT	CTG	CTG	AAC	TAC
C	F	R	H	L	D	E	R	E	E	C	K	C	L	L	N	Y
AAA	CAG	GAG	GGC	GAC	AAG	TGC	GTG	GAG	AAC	CCC	AAC	CCG	ACC	TGT	AAC	GAG
K	Q	E	G	D	K	C	V	E	N	P	N	P	T	C	N	E
AAC	AAC	GGC	GGC	TGT	GAC	GCA	GAC	GCC	AAA	TGC	ACC	GAG	GAG	GAC	TCG	GGC
N	N	G	G	C	D	A	D	A	K	C	T	E	E	D	S	G
AGC	AAC	GGC	AAG	AAA	ATC	ACG	TGT	GAG	TGT	ACC	AAA	CCC	GAC	TCG	TAC	CCG
S	N	G	K	K	I	T	C	E	C	T	K	P	D	S	Y	P
CTG	TTC	GAC	GGC	ATC	TTC	TGC	AGC	TAA	TAA							
L	F	D	G	I	F	C	S	*	*							

FIGURE 1D

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R NR  
P219-A  
G12



FIGURE 2

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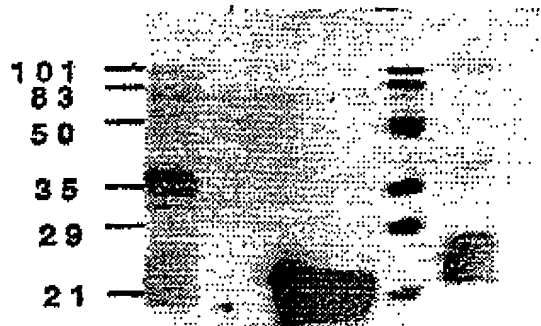
PCT/FR97/00290

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MSP1p19S

V

C



NRIR R

NRIR R

FIGURE 2B

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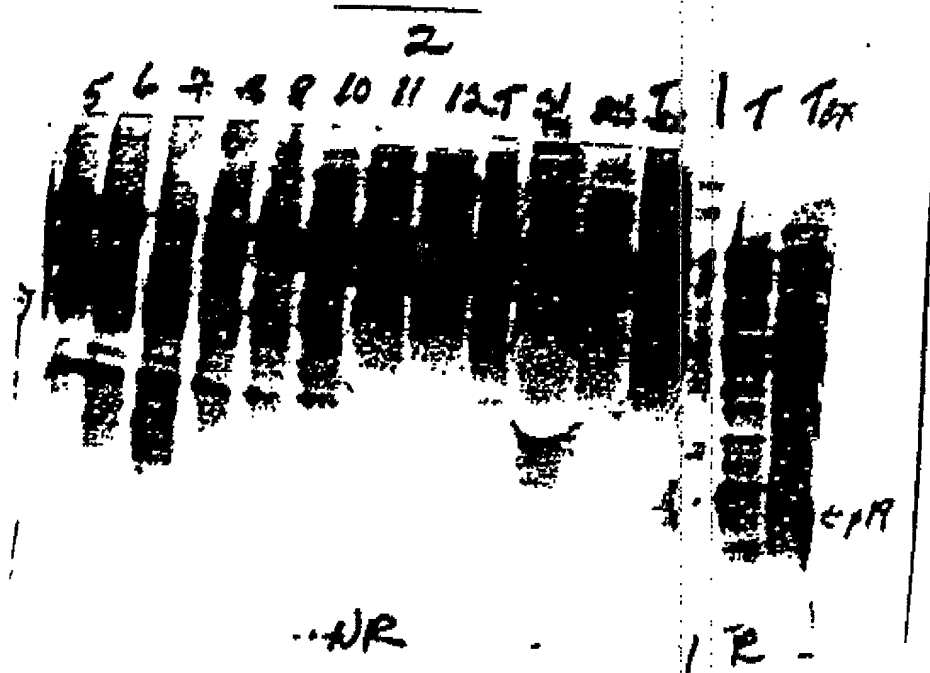


FIGURE 3

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SECRET

ELISA INHIBITION BY P. VIVAX MSP-1 P42 AND P19 ANTIGENS

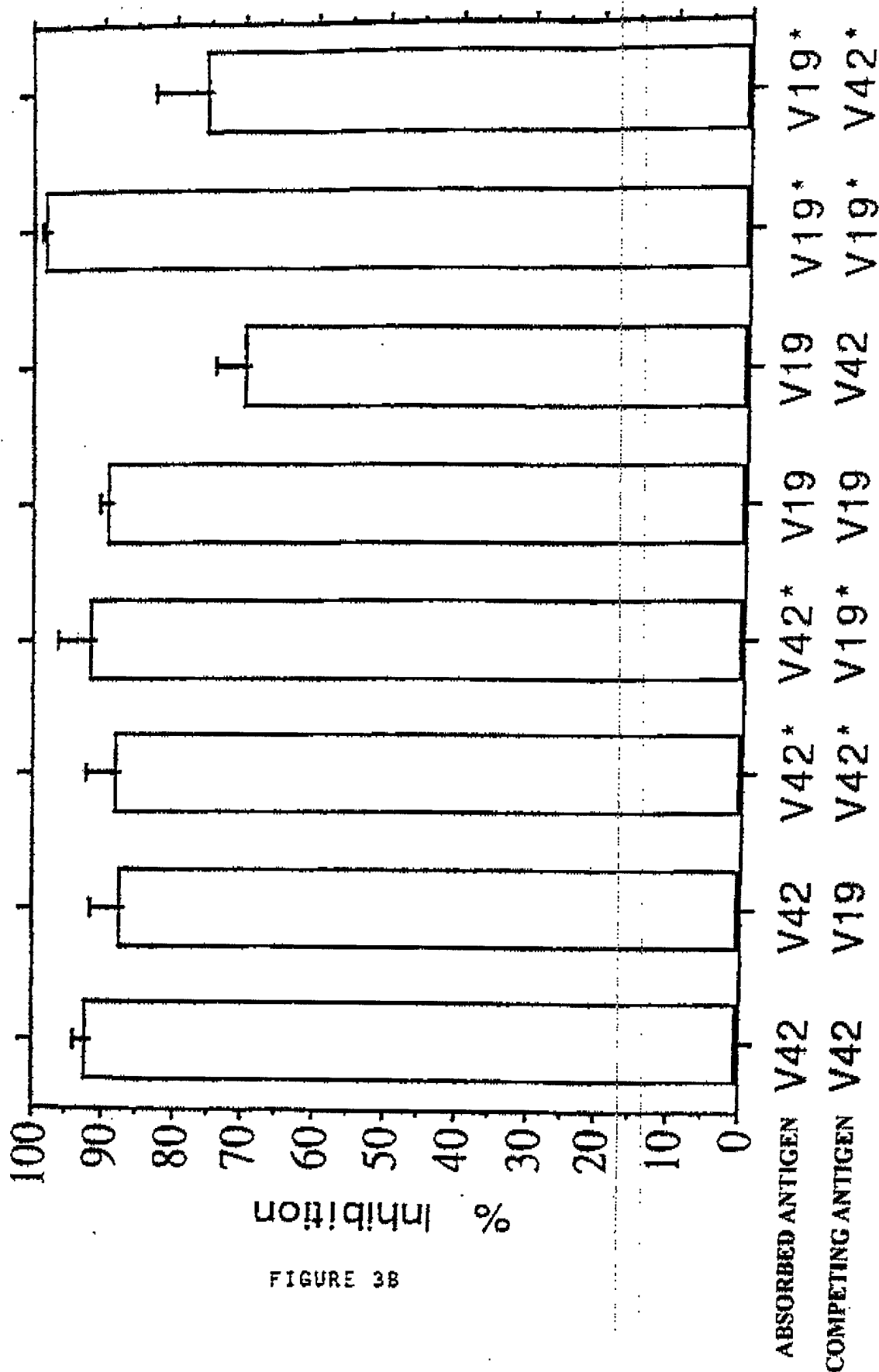


FIGURE 38



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SEQUENCE FIGURE 4

A

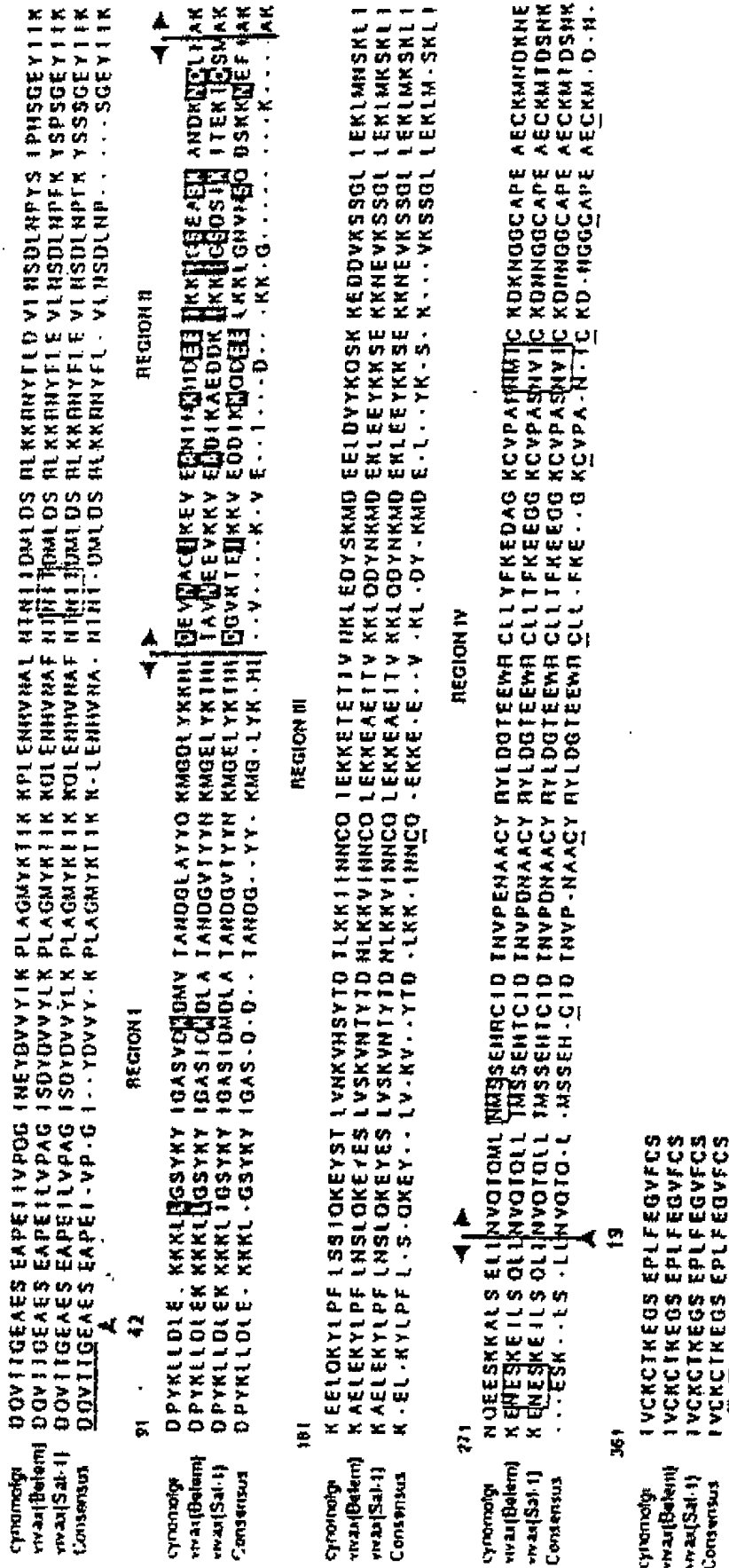


FIGURE 4

B

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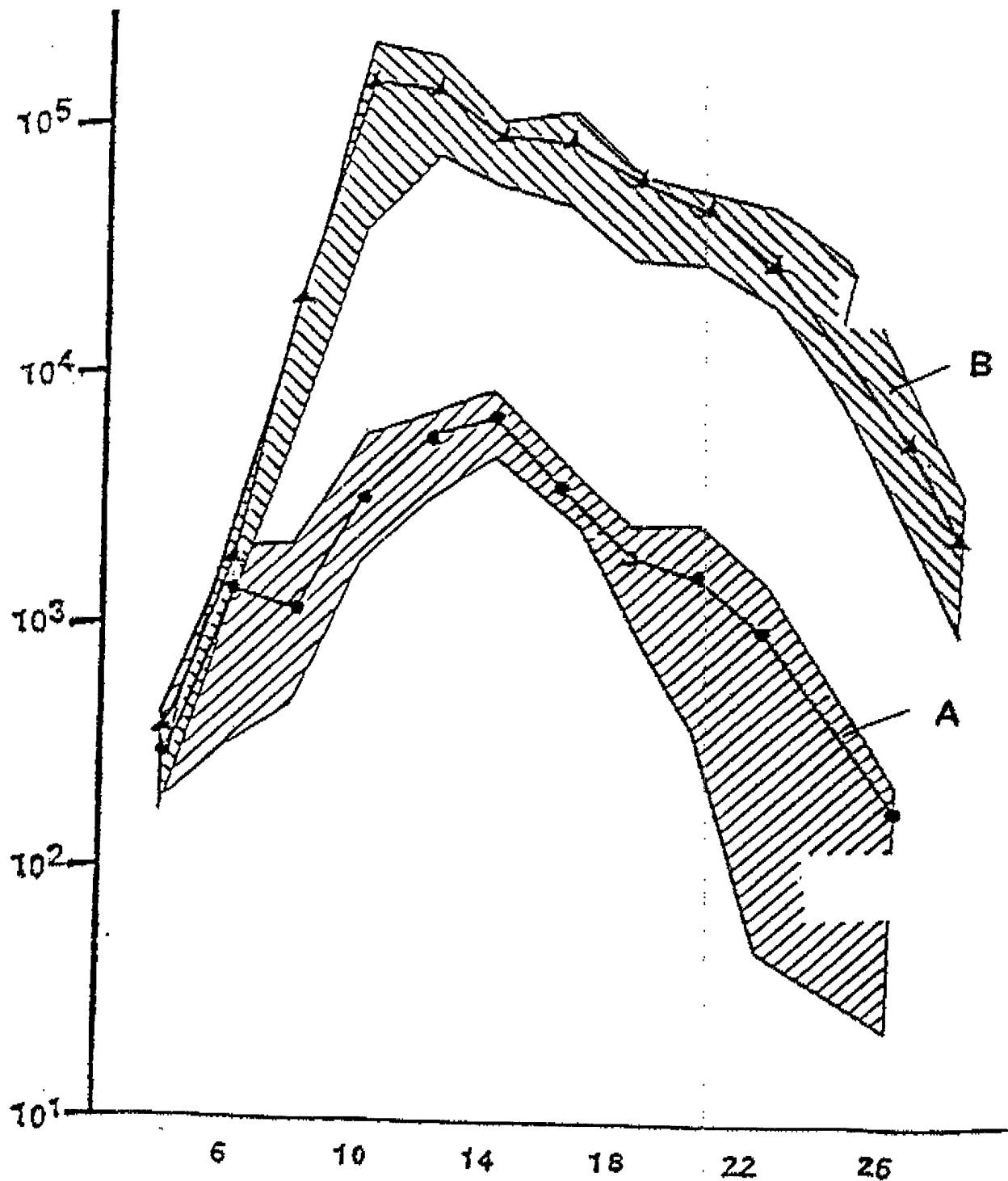


FIGURE 5

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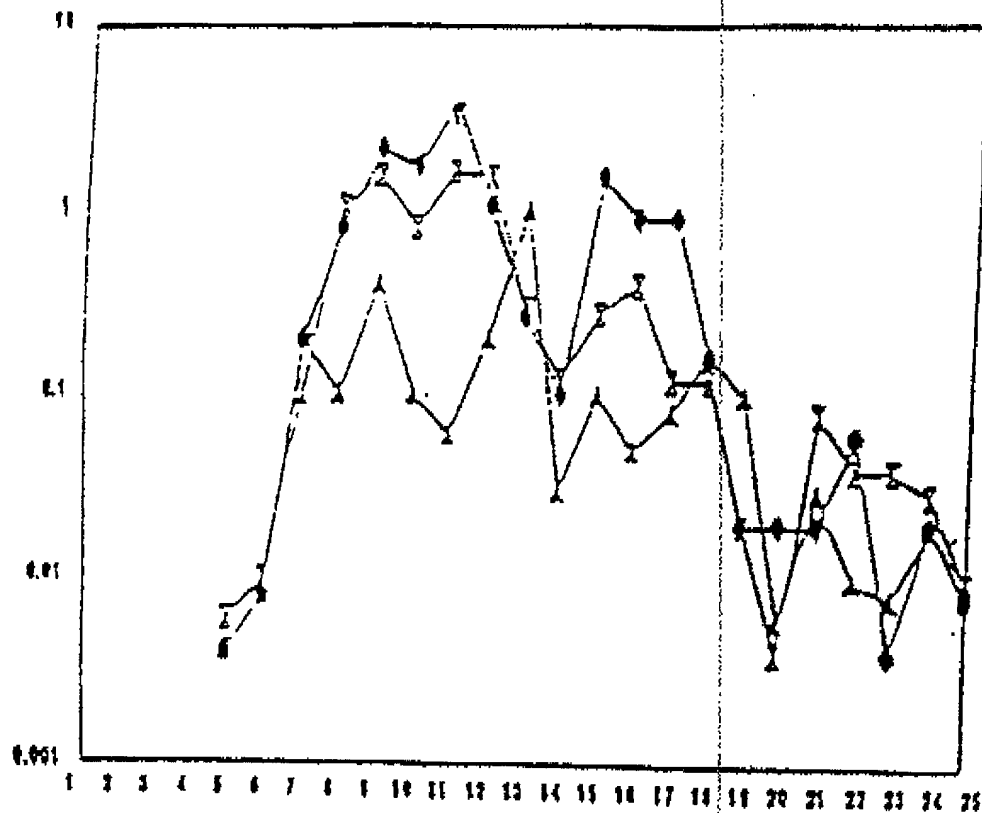


FIGURE 6A

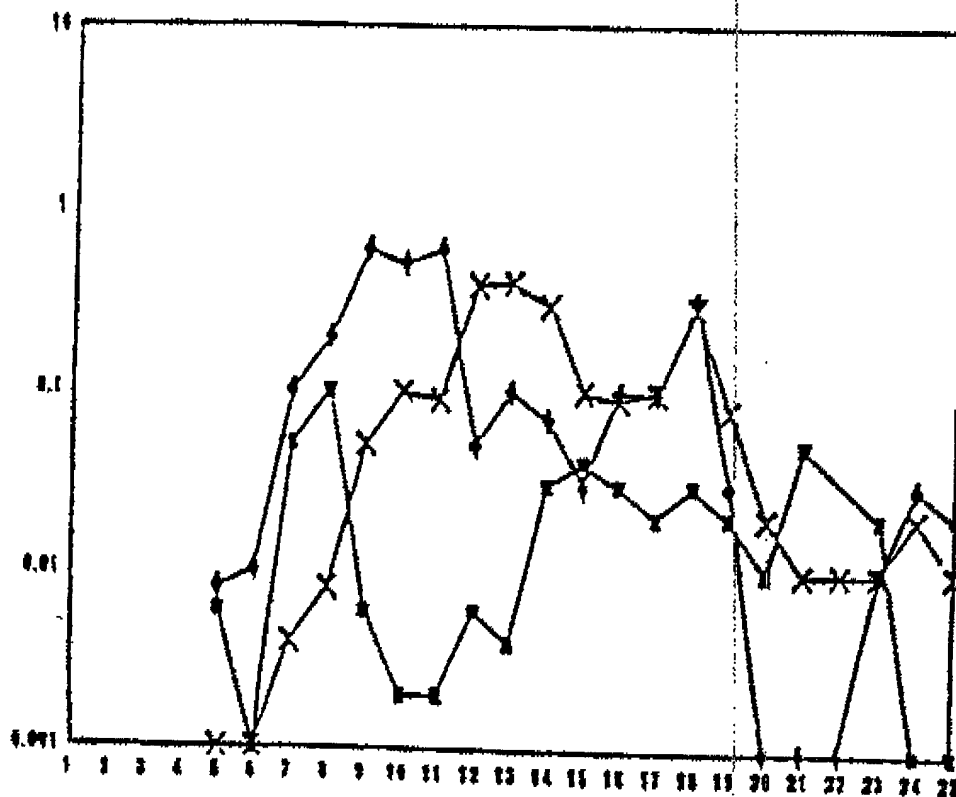


FIGURE 6B

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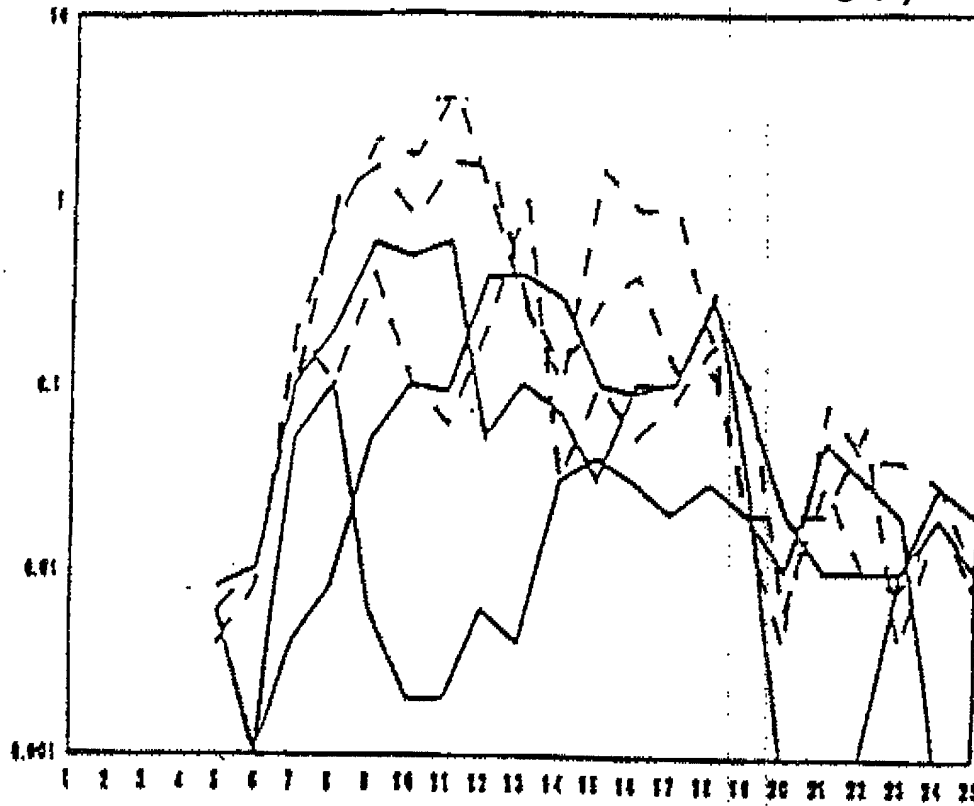


FIGURE 6C

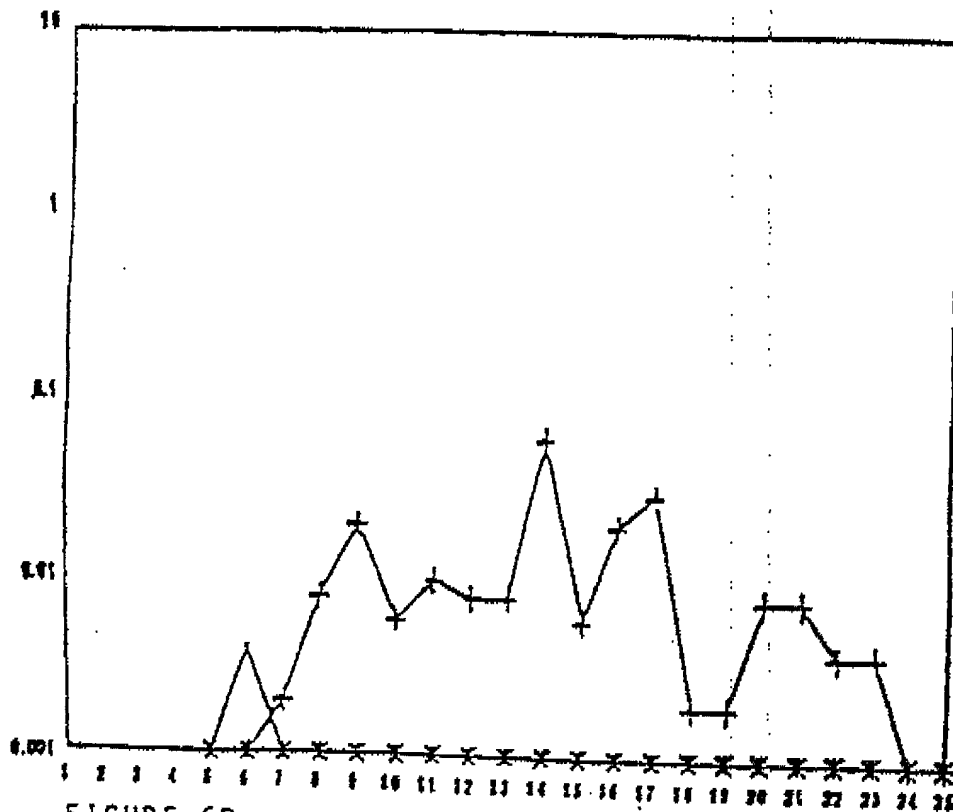


FIGURE 6D

600720 70022760



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FIGURE 6G

[illegible][illegible][illegible][illegible][illegible][illegible]

Controls	Non vaccinated	1437	1440	1441	1442	1443	1444	1445	1446	1447	1448	1449	1450	1451	1452	1453	1454	1455	1456	1457	1458	1459	1460	1461	1462	1463	1464	1465	1466	1467	1468	1469	1470	1471	1472	1473	1474	1475	1476	1477	1478	1479	1480	1481	1482	1483	1484	1485	1486	1487	1488	1489	1490	1491	1492	1493	1494	1495	1496	1497	1498	1499
Controls	Non vaccinated	1437	1440	1441	1442	1443	1444	1445	1446	1447	1448	1449	1450	1451	1452	1453	1454	1455	1456	1457	1458	1459	1460	1461	1462	1463	1464	1465	1466	1467	1468	1469	1470	1471	1472	1473	1474	1475	1476	1477	1478	1479	1480	1481	1482	1483	1484	1485	1486	1487	1488	1489	1490	1491	1492	1493	1494	1495	1496	1497	1498	1499

— = Absence of parasites in 400 microscopic fields

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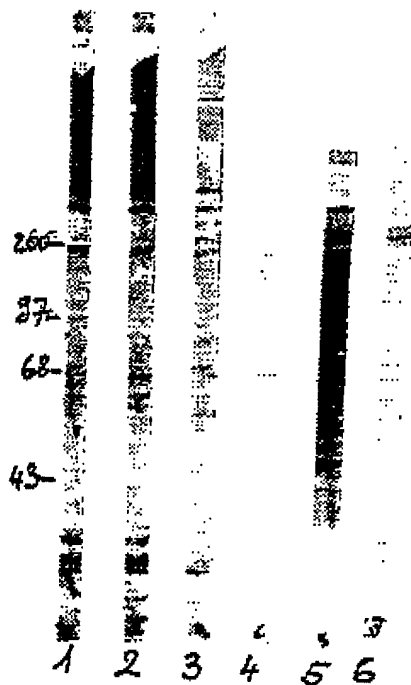


FIGURE 7

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660760760

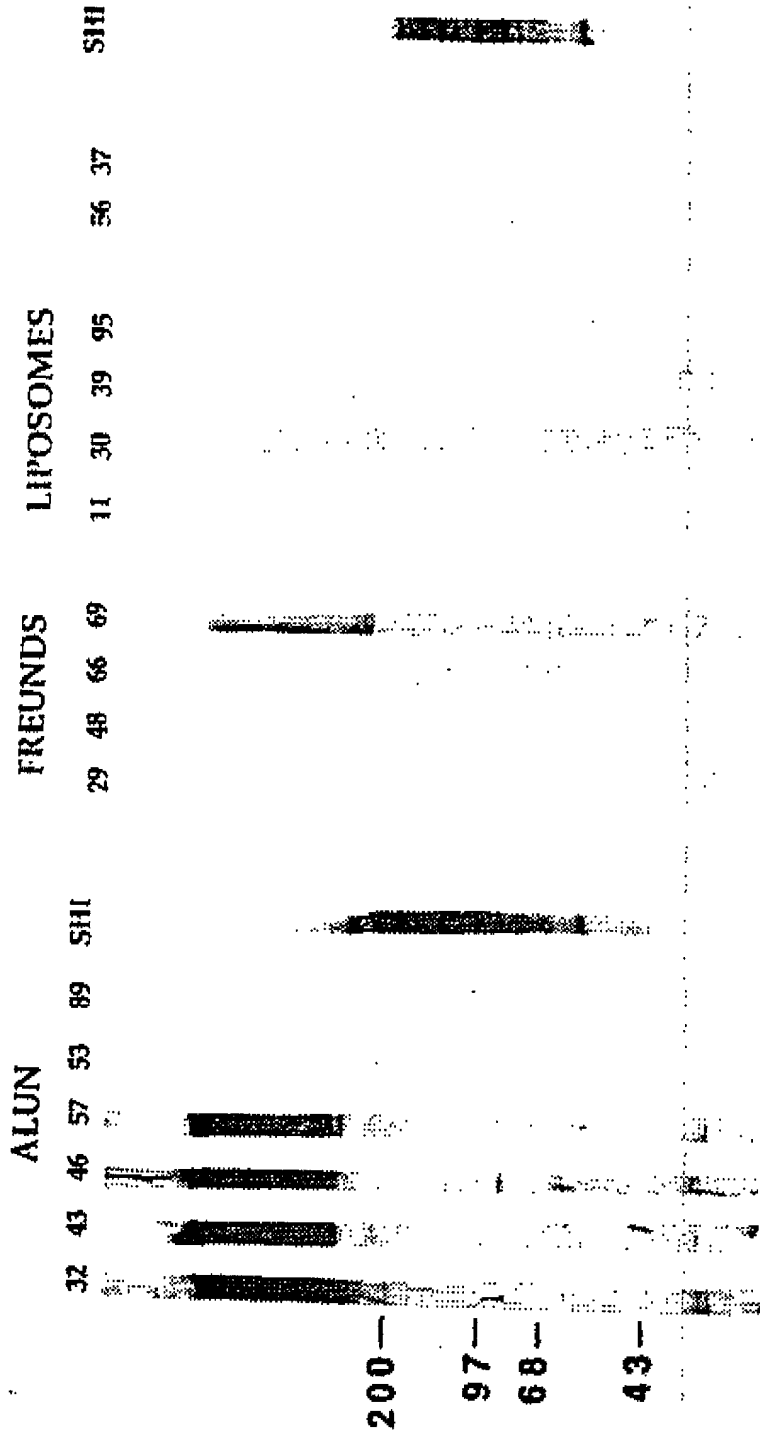


FIGURE 7B



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FIGURE 8A

Vaccination test: recombinant MSP-1 (p19) of *Plasmodium cynomolgi* in the *Macaca sinica* toque  
macaque; second challenge infection

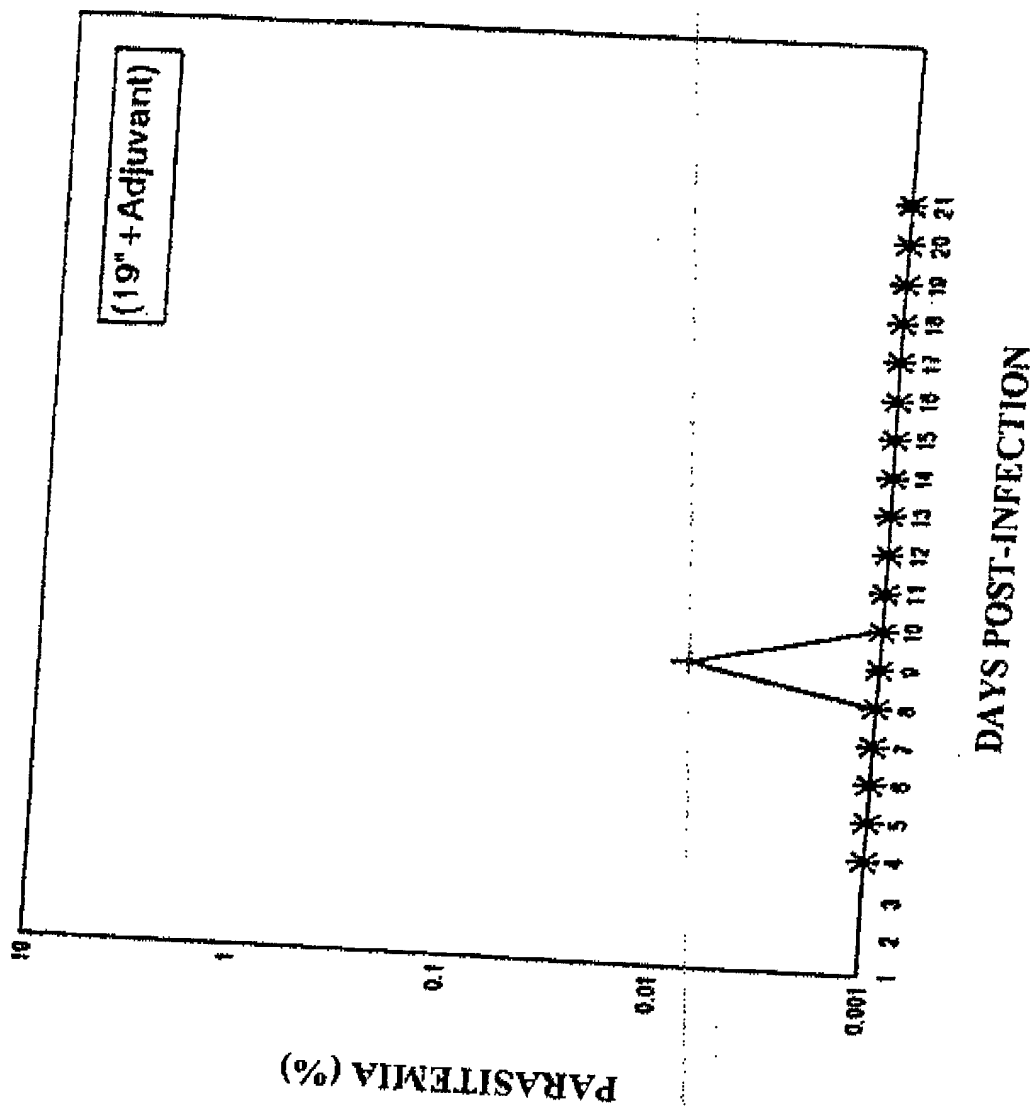
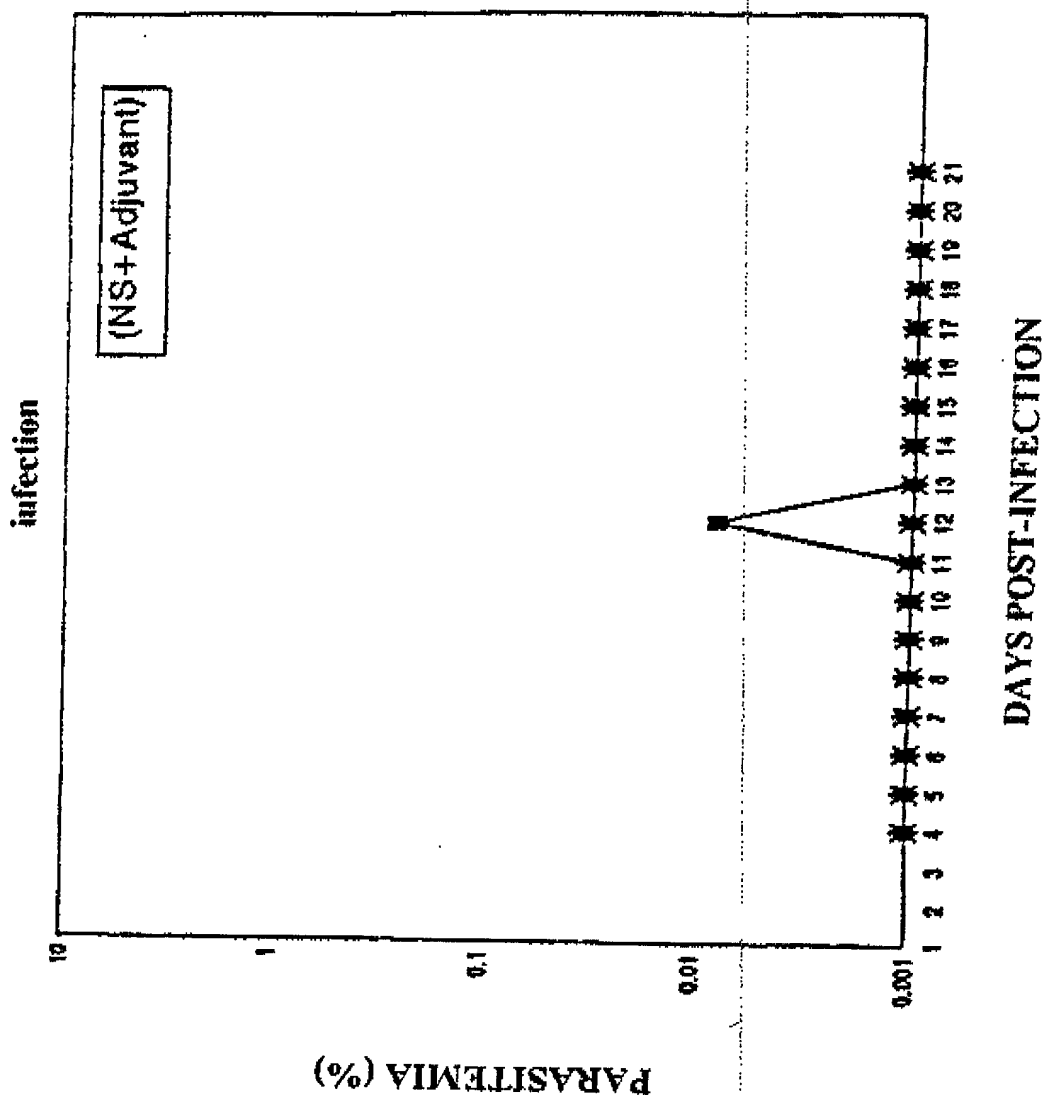


FIGURE 8B

Vaccination test: recombinant MSP-1 (p19) of *Plasmodium cynomolgi* in the *Macaca sinica* toque macaque; second challenge



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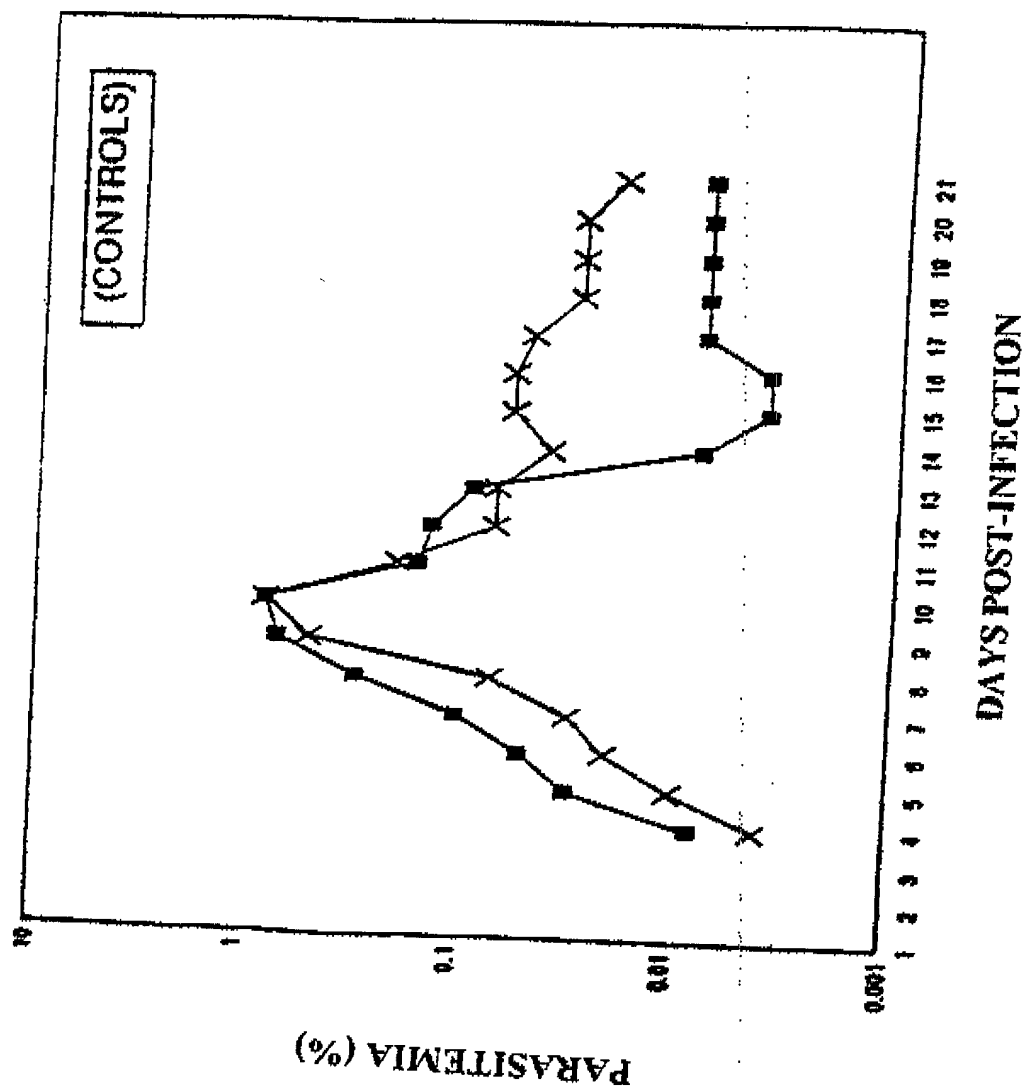
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FIGURE 8C



650760-TE02750  
 Vaccination test: recombinant MSP-1 (p19) of *Plasmodium cynomolgi* in the *Macaca sinica* toque macaque; second challenge

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Vaccination test: recombinant MSP-1 (p19) of *Plasmodium cynomolgi* in the*Macaca sinica* toque macaque: second challenge infection

Year	96	96	96	96	96	96	96	96	96	96	96	96	96	96	96	96	96	96
Month	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2
Days post-infection	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20
Days after challenge	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21
Vaccination p19																		
T 426	-	-	-	-	-	.008	-	-	-	-	-	-	-	-	-	-	-	-
T 427	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
T 429	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Controls Physiological water FCA/FIA																		
T 436	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
T 425	-	-	-	-	-	-	-	-	.008	-	-	-	-	-	-	-	-	-
T 438	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Controls Non vaccinated																		
T 448	.008	.03	.05	0.1	0.3	0.7	0.8	.16	.14	.09	.008	.004	.004	.008	.008	.008	.008	.008
T 449	.004	.01	.02	.03	0.07	0.5	0.8	0.2	.07	.07	.04	.06	.06	.05	.03	.03	.03	.02

- = Absence of parasites in 400 microscopic fields

FIGURE 8D

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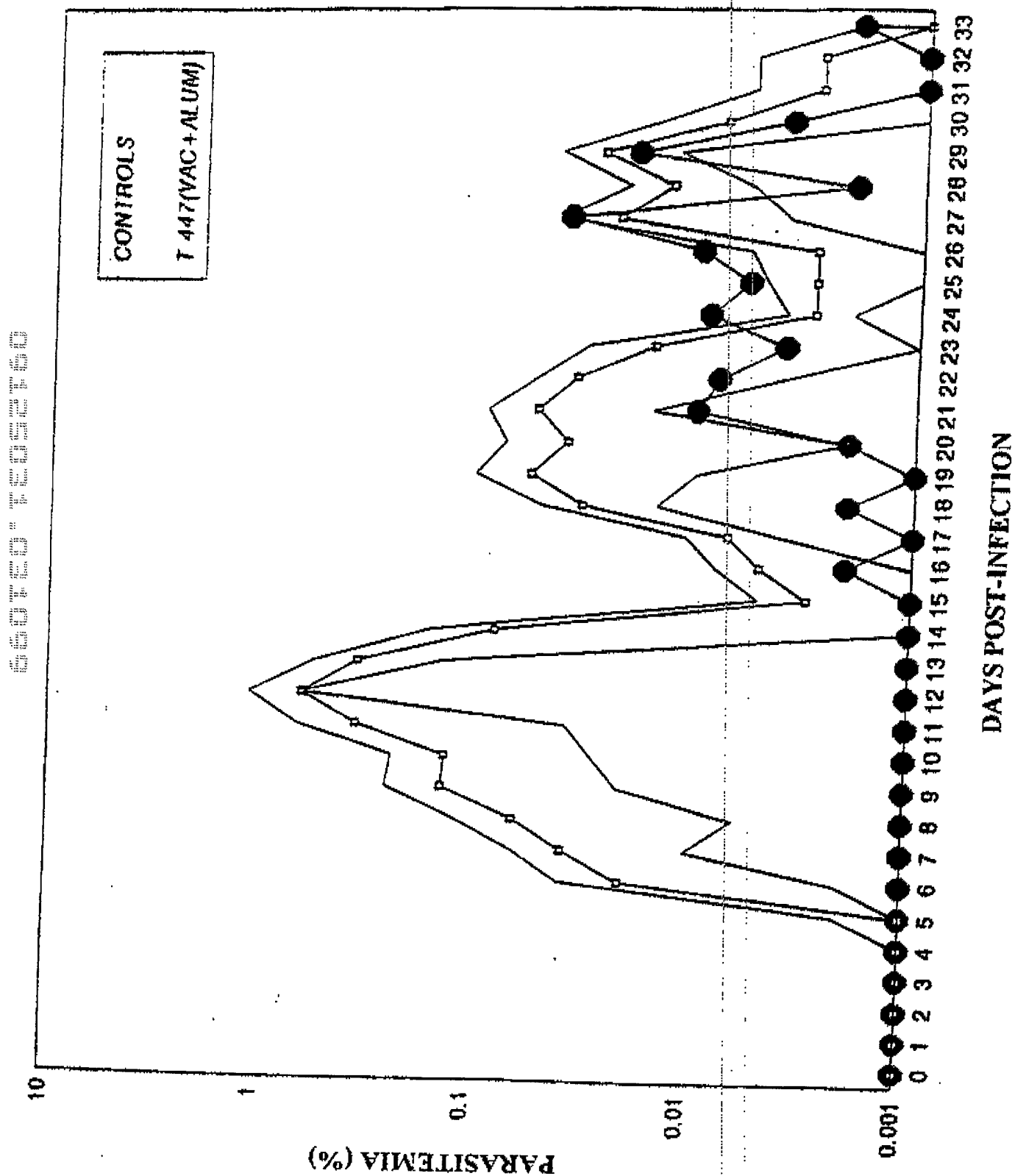
DOCKET # 84009/390XPG SHEET 21 OF 32

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FIGURE 9A



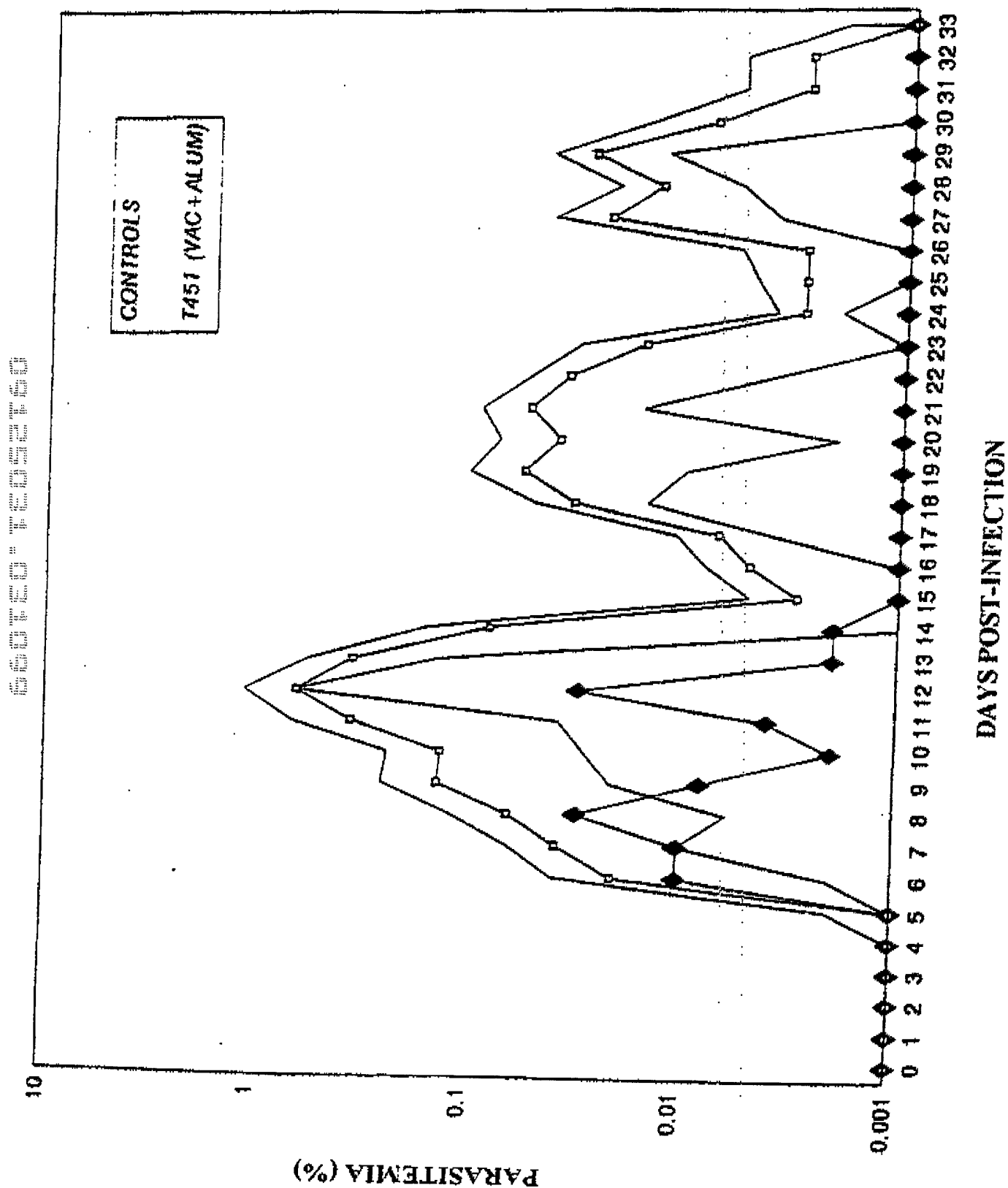
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FIGURE 9B



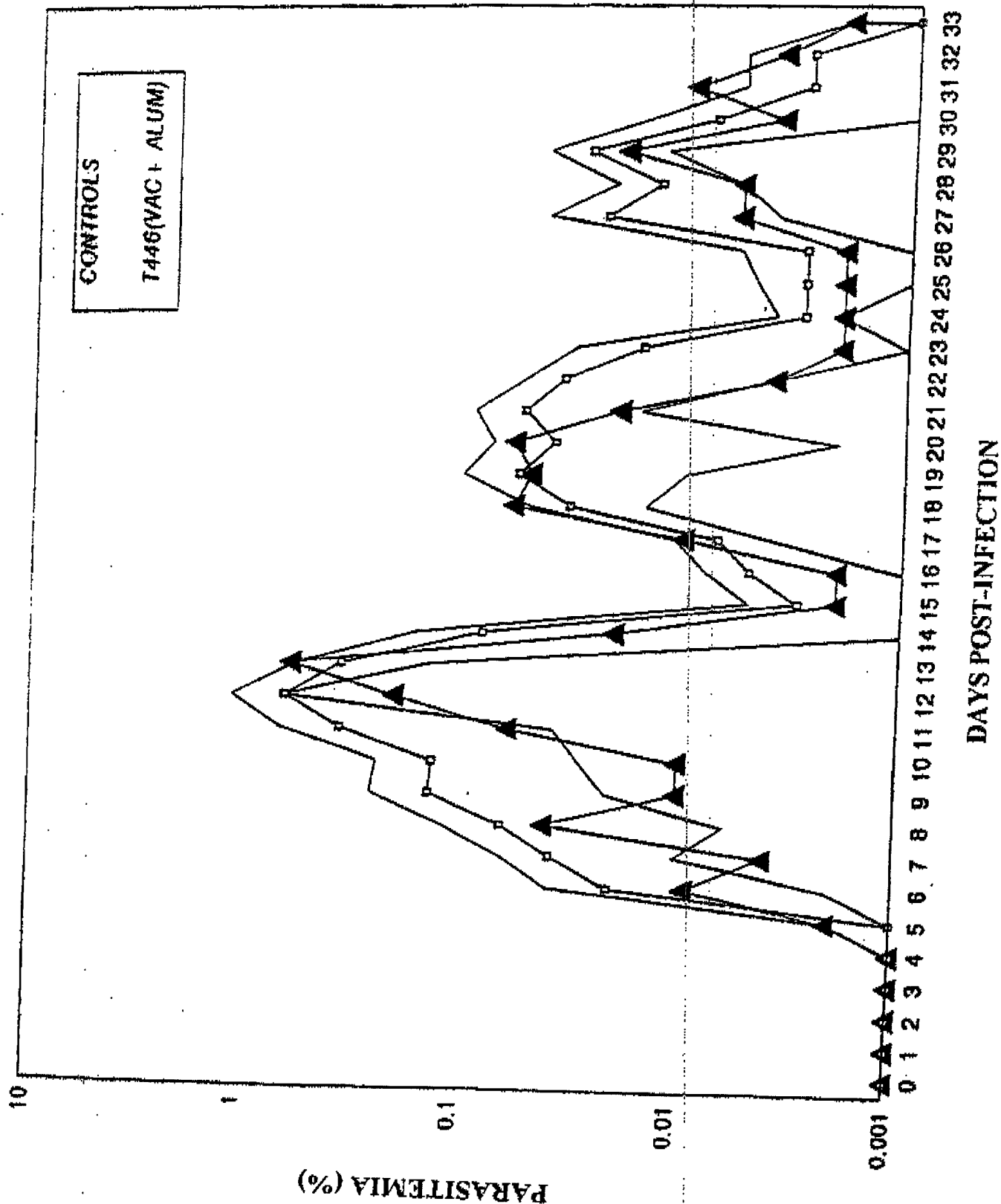
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FIGURE 9C



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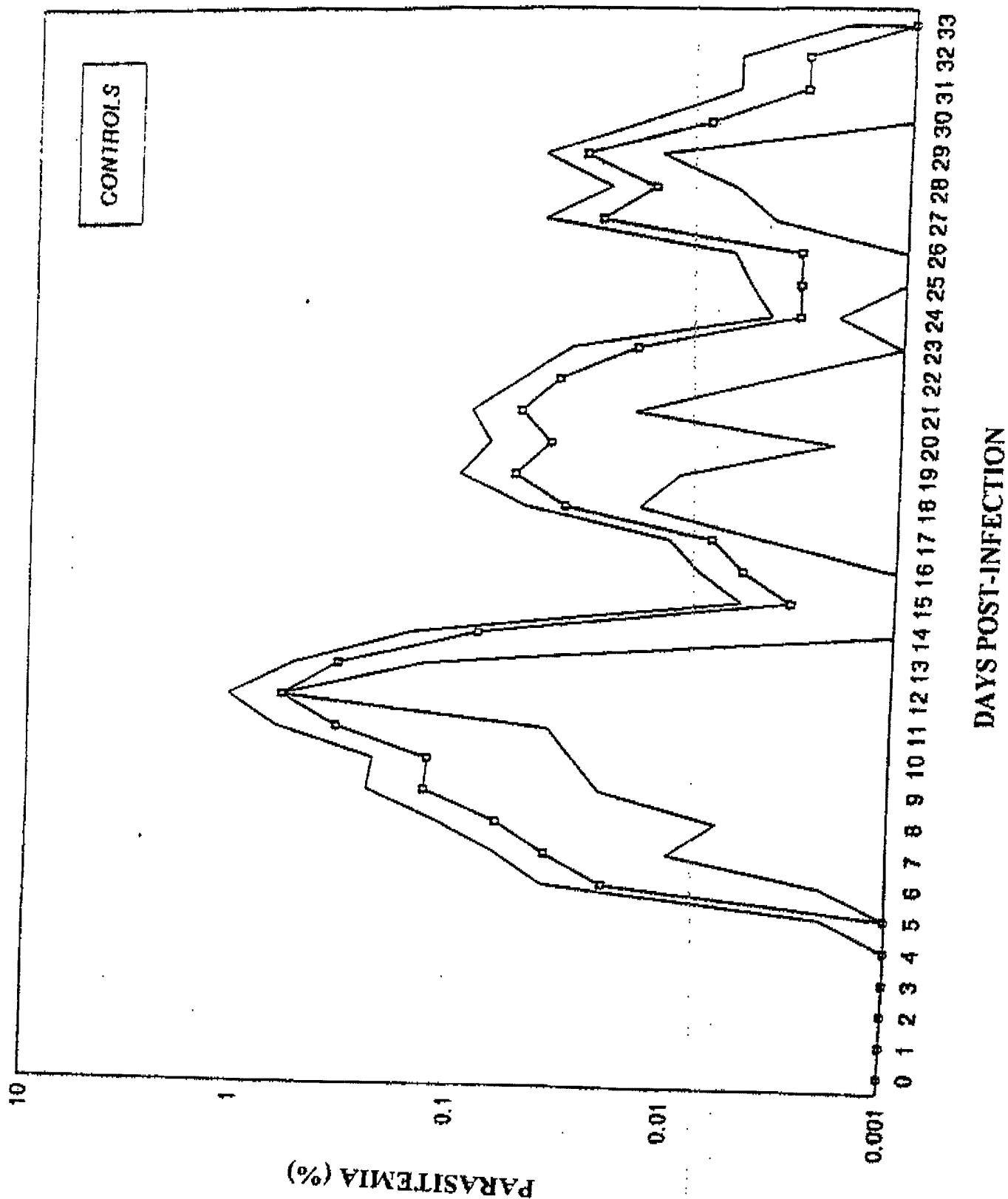
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FIGURE 9D





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Vaccination test: *P. cynomolgi*/toque macaque with MSP-1 p19 of *P.**cynomolgi* in alum

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Year  
Month  
Day

GROUP 1  
(HS + ALUM)

T 446

T 447

T 451

GROUP 2  
(NS + ALUM)

T 439

T 454

T 455

98	96	96	96	96	96	96	96	96	96	96	96	96	96	96	96	96	96	96	96	96	96	96	96	96	96	96	96	96	96	96	96	96	96	96	96	96	96	96	96	96	96	96	96	96	96	96	96	96	96	96	96	96	96	96	96	96	96	96	96	96	96	96	96	96	96	96	96	96	96	96	96	96	96	96	96	96	96	96	96	96	96	96	96	96	96	96	96	96	96	96	96	96	96	96	96	96	96	96	96	96	96	96	96	96	96	96	96	96	96	96	96	96	96	96	96	96	96	96	96	96	96	96	96	96	96	96	96	96	96	96	96	96	96	96	96	96	96	96	96	96	96	96	96	96	96	96	96	96	96	96	96	96	96	96	96	96	96	96	96	96	96	96	96	96	96	96	96	96	96	96	96	96	96	96	96	96	96	96	96	96	96	96	96	96	96	96	96	96	96	96	96	96	96	96	96	96	96	96	96	96	96	96	96	96	96	96	96	96	96	96	96	96	96	96	96	96	96	96	96	96	96	96	96	96	96	96	96	96	96	96	96	96	96	96	96	96	96	96	96	96	96	96	96	96	96	96	96	96	96	96	96	96	96	96	96	96	96	96	96	96	96	96	96	96	96	96	96	96	96	96	96	96	96	96	96	96	96	96	96	96	96	96	96	96	96	96	96	96	96	96	96	96	96	96	96	96	96	96	96	96	96	96	96	96	96	96	96	96	96	96	96	96	96	96	96	96	96	96	96	96	96	96	96	96	96	96	96	96	96	96	96	96	96	96	96	96	96	96	96	96	96	96	96	96	96	96	96	96	96	96	96	96	96	96	96	96	96	96	96	96	96	96	96	96	96	96	96	96	96	96	96	96	96	96	96	96	96	96	96	96	96	96	96	96	96	96	96	96	96	96	96	96	96	96	96	96	96	96	96	96	96	96	96	96	96	96	96	96	96	96	96	96	96	96	96	96	96	96	96	96	96	96	96	96	96	96	96	96	96	96	96	96	96	96	96	96	96	96	96	96	96	96	96	96	96	96	96	96	96	96	96	96	96	96	96	96	96	96	96	96	96	96	96	96	96	96	96	96	96	96	96	96	96	96	96	96	96	96	96	96	96	96	96	96	96	96	96	96	96	96	96	96	96	96	96	96	96	96	96	96	96	96	96	96	96	96	96	96	96	96	96	96	96	96	96	96	96	96	96	96	96	96	96	96	96	96	96	96	96	96	96	96	96	96	96	96	96	96	96	96	96	96	96	96	96	96	96	96	96	96	96	96	96	96	96	96	96	96	96	96	96	96	96	96	96	96	96	96	96	96	96	96	96	96	96	96	96	96	96	96	96	96	96	96	96	96	96	96	96	96	96	96	96	96	96	96	96	96	96	96	96	96	96	96	96	96	96	96	96	96	96	96	96	96	96	96	96	96	96	96	96	96	96	96	96	96	96	96	96	96	96	96	96	96	96	96	96	96	96	96	96	96	96	96	96	96	96	96	96	96	96	96	96	96	96	96	96	96	96	96	96	96	96	96	96	96	96	96	96	96	96	96	96	96	96	96	96	96	96	96	96	96	96	96	96	96	96	96	96	96	96	96	96	96	96	96	96	96	96	96	96	96	96	96	96	96	96	96	96	96	96	96	96	96	96	96	96	96	96	96	96	96	96	96	96	96	96	96	96	96	96	96	96	96	96	96	96	96	96	96	96	96	96	96	96	96	96	96	96	96	96	96	96	96	96	96	96	96	96	96	96	96	96	96	96	96	96	96	96	96	96	96	96	96	96	96	96	96	96	96	96	96	96	96	96	96	96	96	96	96	96	96	96	96	96	96	96	96	96	96	96	96	96	96	96	96	96	96	96	96	96	96	96	96	96	96	96	96	96	96	96	96	96	96	96	96	96	96	96	96	96	96	96	96	96	96	96	96	96	96	96	96	96	96	96	96	96	96	96	96	96	96	96	96	96	96	96	96	96	96	96	96	96	96	96	96	96	96	96	96	96	96	96	96	96	96	96	96	96	96	96	96	96	96	96	96	96	96	96	96	96	96	96	96	96	96	96	96	96	96	96	96	96	96	96	96	96	96	96	96	96	96	96	96	96	96	96	96	96	96	96	96	96	96	96	96	96	96	96	96	96	96	96	96	96	96	96	96	96	96	96	96	96	96	96	96	96	96	96	96	96	96	96	96	96	96	96	96	96	96	96	96	96	96	96	96	96	96	96	96	96	96	96	96	96	96	96	96	96	96	96	96	96	96	96	96	96	96	96	96	96	96	96	96	96	96	96	96	96	96	96	96	96	96	96	96	96	96	96	96	96	96	96	96	96	96	96	96	96	96	96	96	96	96	96	96	96	96	96	96	96	96	96	96	96	96	96	96	96	96	96	96	96	96	96	96	96
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GROUP 1  
(15 + ALUM)

T446

T447

T451

GROUP 2  
(NS + ALUM)

T450

T454

T455

FIGURE 9E

— negative for parasites in 400 microscopic fields

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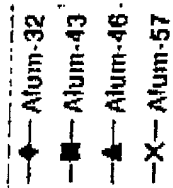
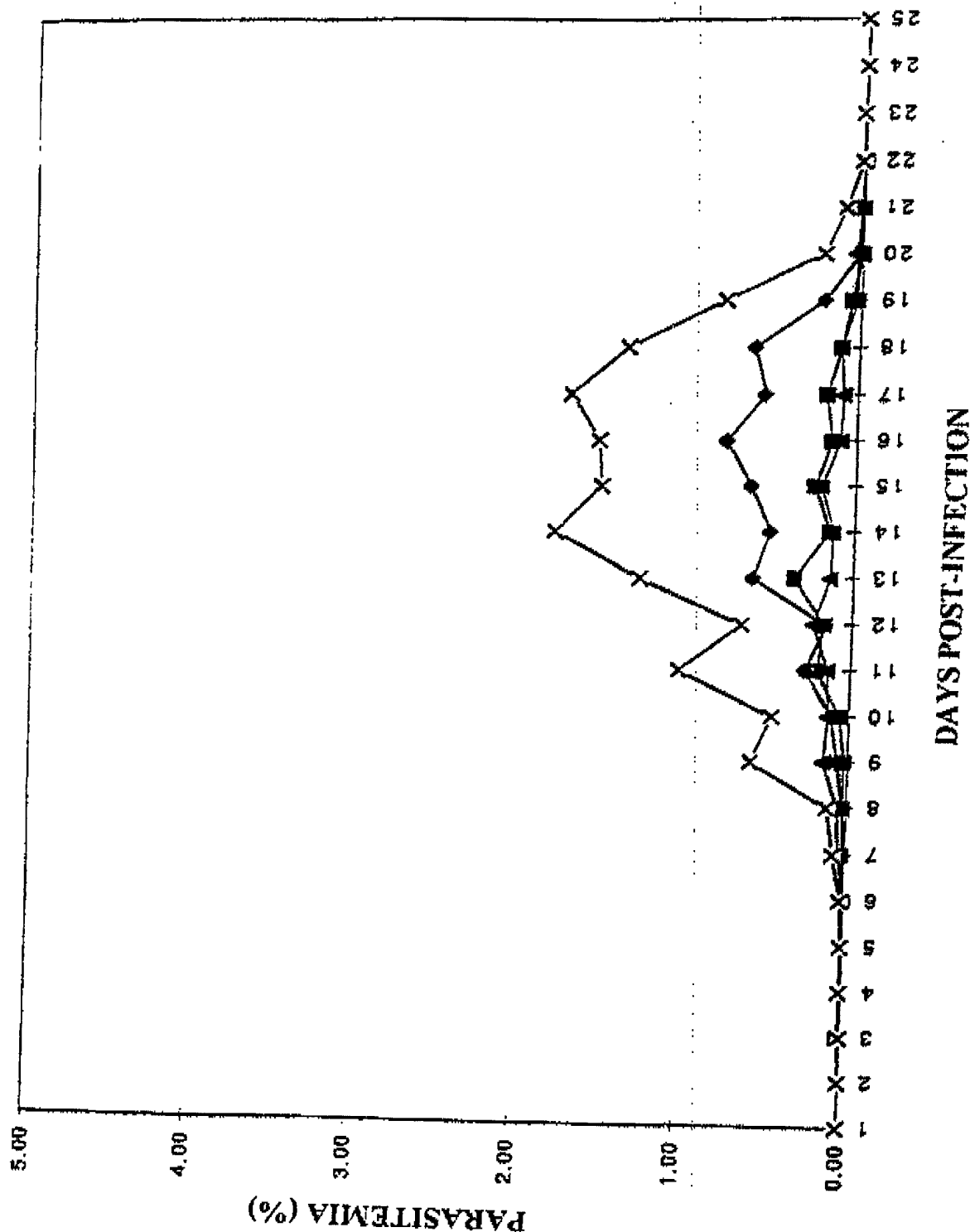


FIGURE 10A

PLASMODIUM FALCIPARUM VACCINATION TEST IN SAHARI  
SCIUREUS SQUIRREL MONKEY  
MSP-1 p19 VACCINATION WITH ALUM



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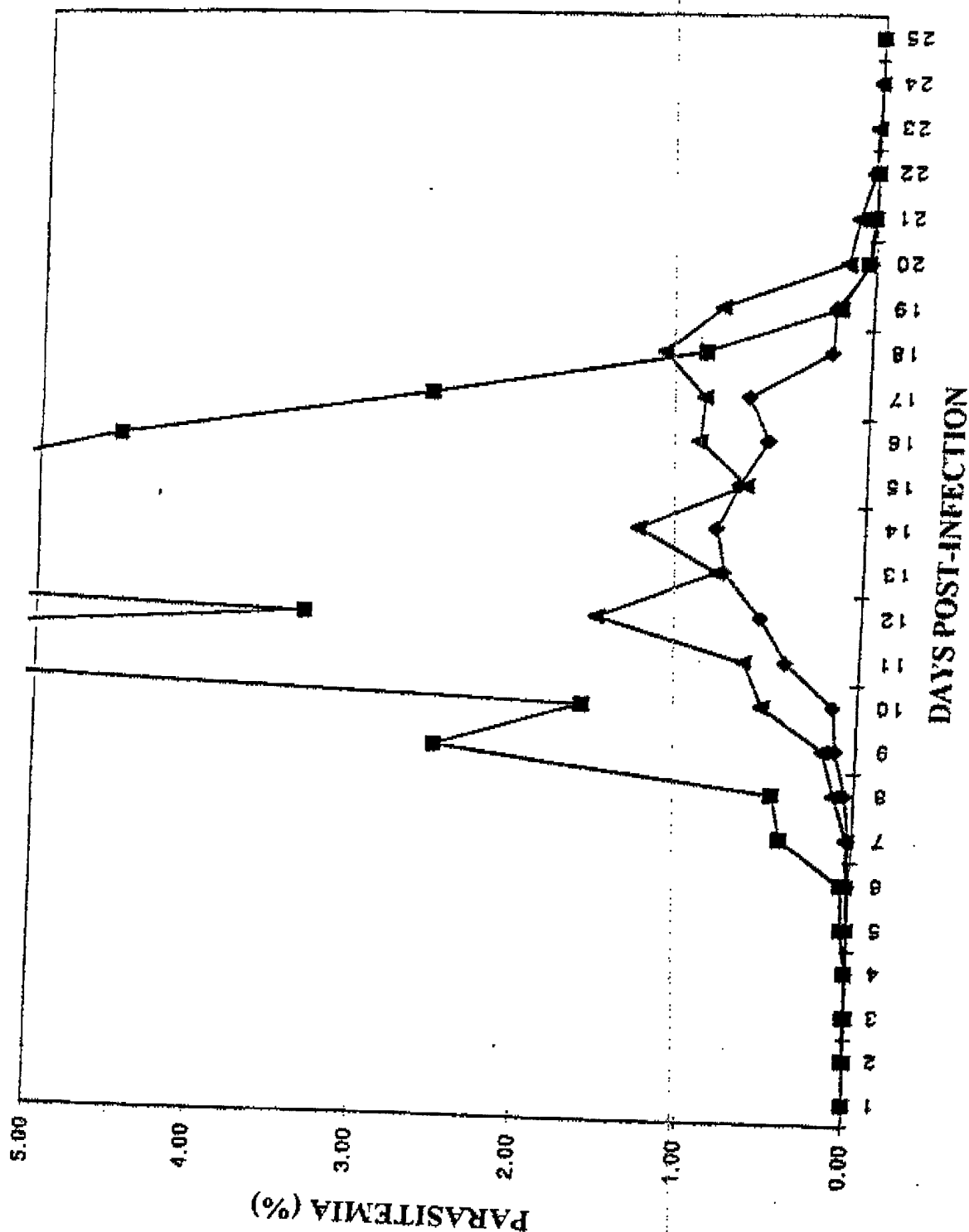
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Freund-48  
Freund-56  
Freund-69

FIGURE 10B



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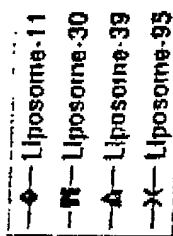
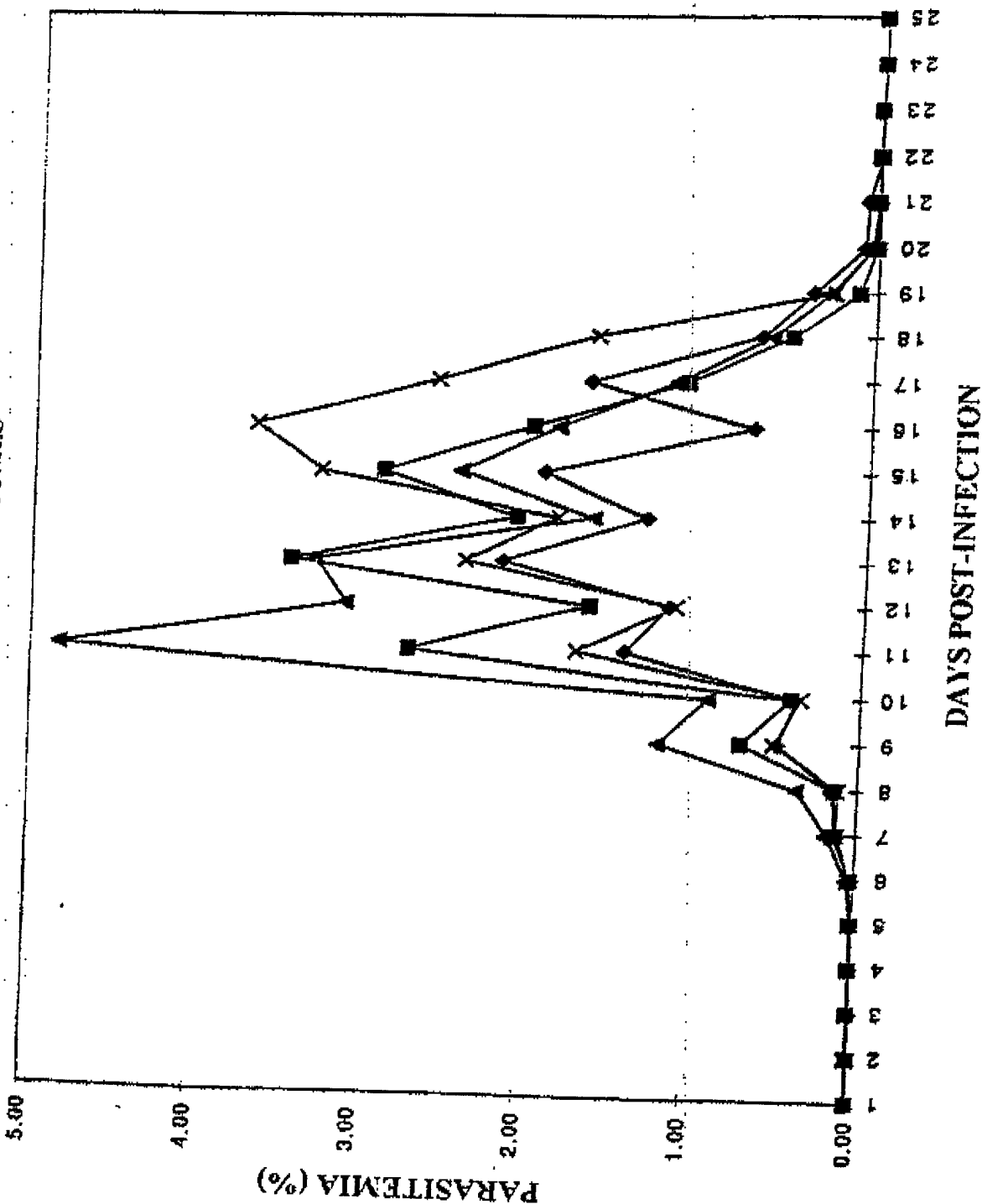


FIGURE 10C



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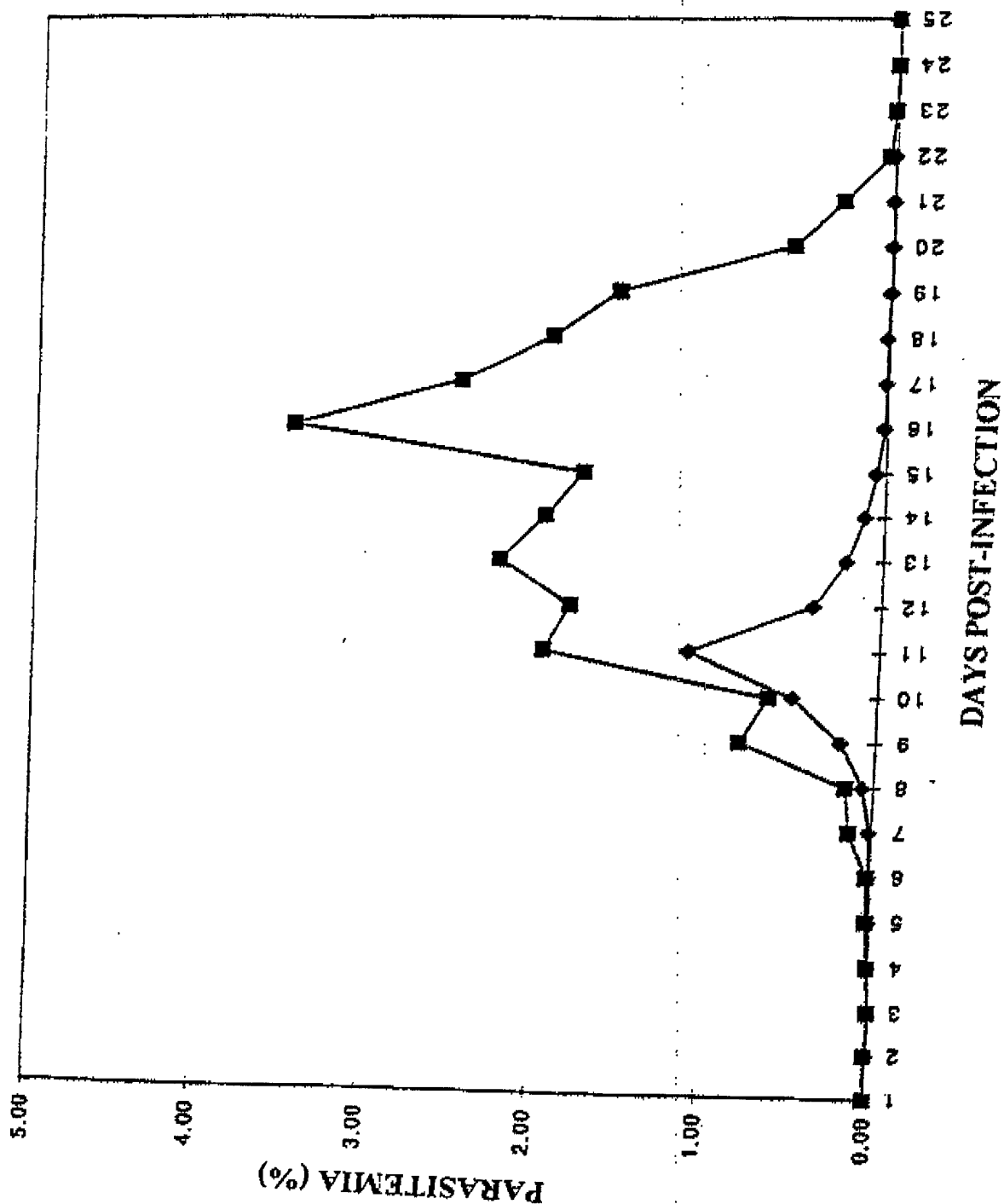
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FIGURE 100

PLASMODIUM FALCIPARUM VACCINATION TEST IN SAIMIRI  
SCIUREUS SQUIRREL MONKEY  
ALUM CONTROL



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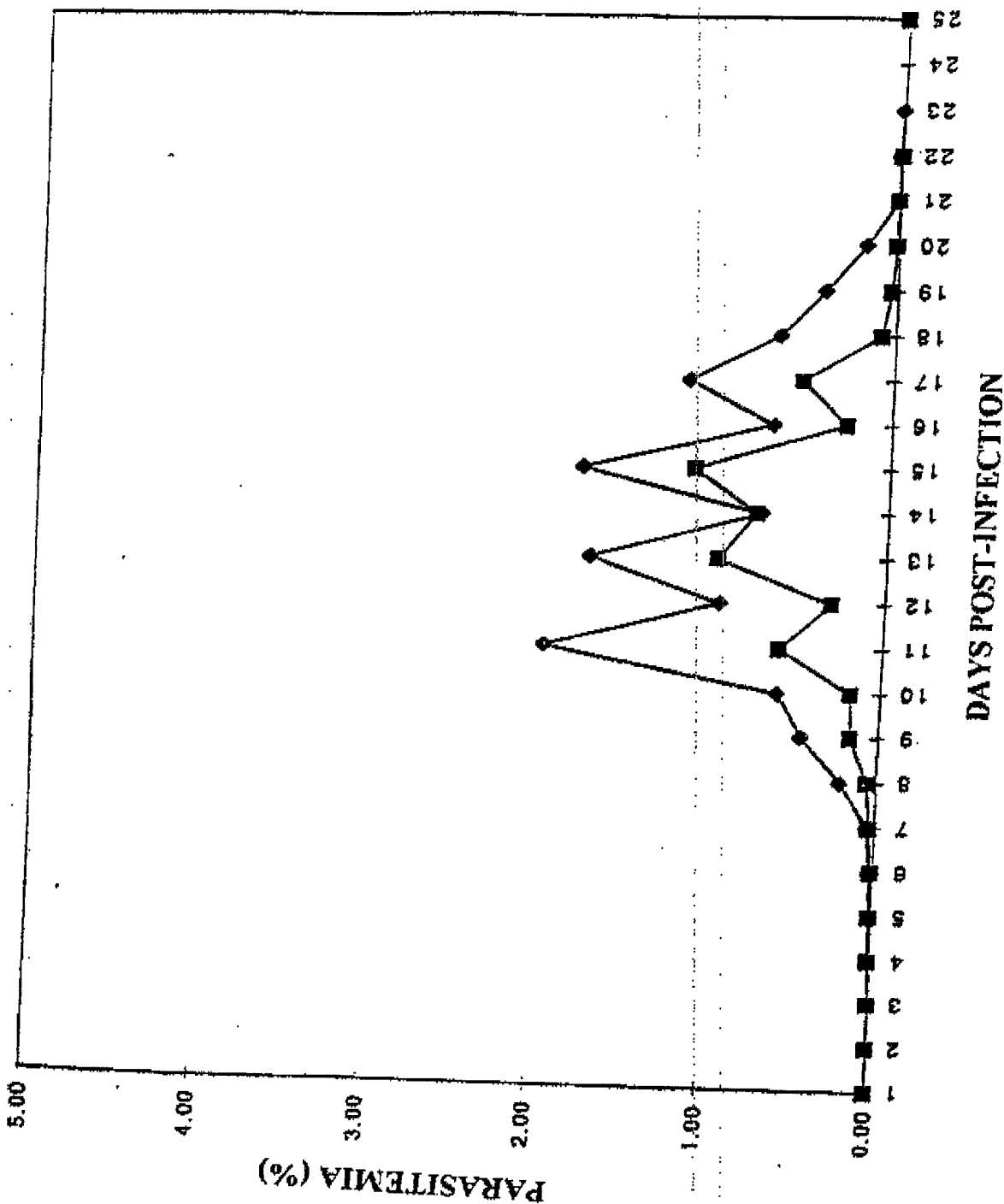
PCT/FR97/00290

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Fr Control-56  
Fr Control-108

FIGURE 10E

PLASMODIUM FALCIPARUM VACCINATION TEST IN SAMIRI  
SCIUREUS SQUIRREL MONKEY  
FREUNDS CONTROL



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SHEET 31 OF 32

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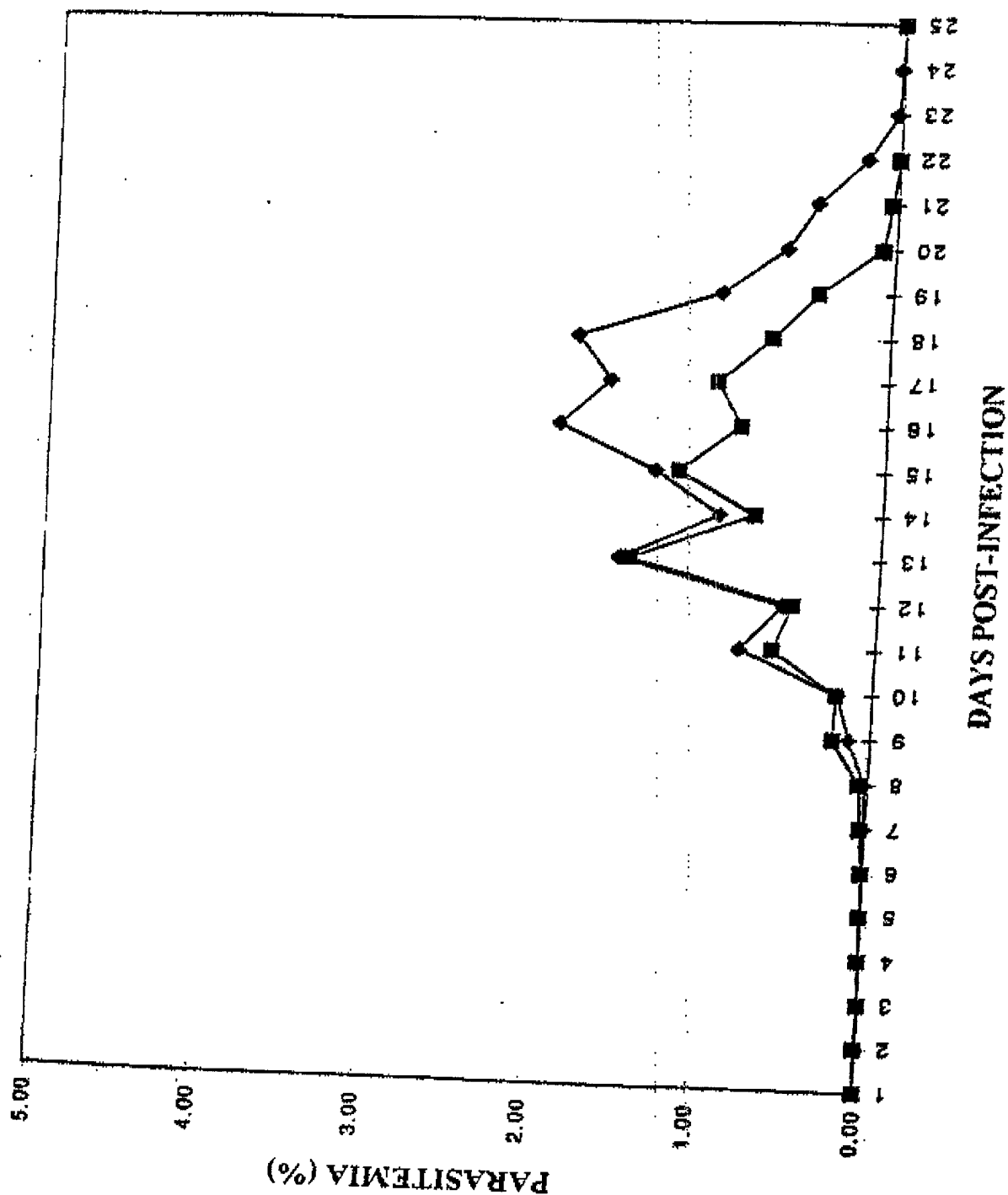
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Lip Control-37  
 Lip Control-73

FIGURE 10F

PLASMODIUM FALCIPARUM VACCINATION TEST IN SAHARI  
 SCIUREUS SQUIRREL MONKEY  
 LIPOSOME CONTROL



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BLON ET AL (703) 413-3000  
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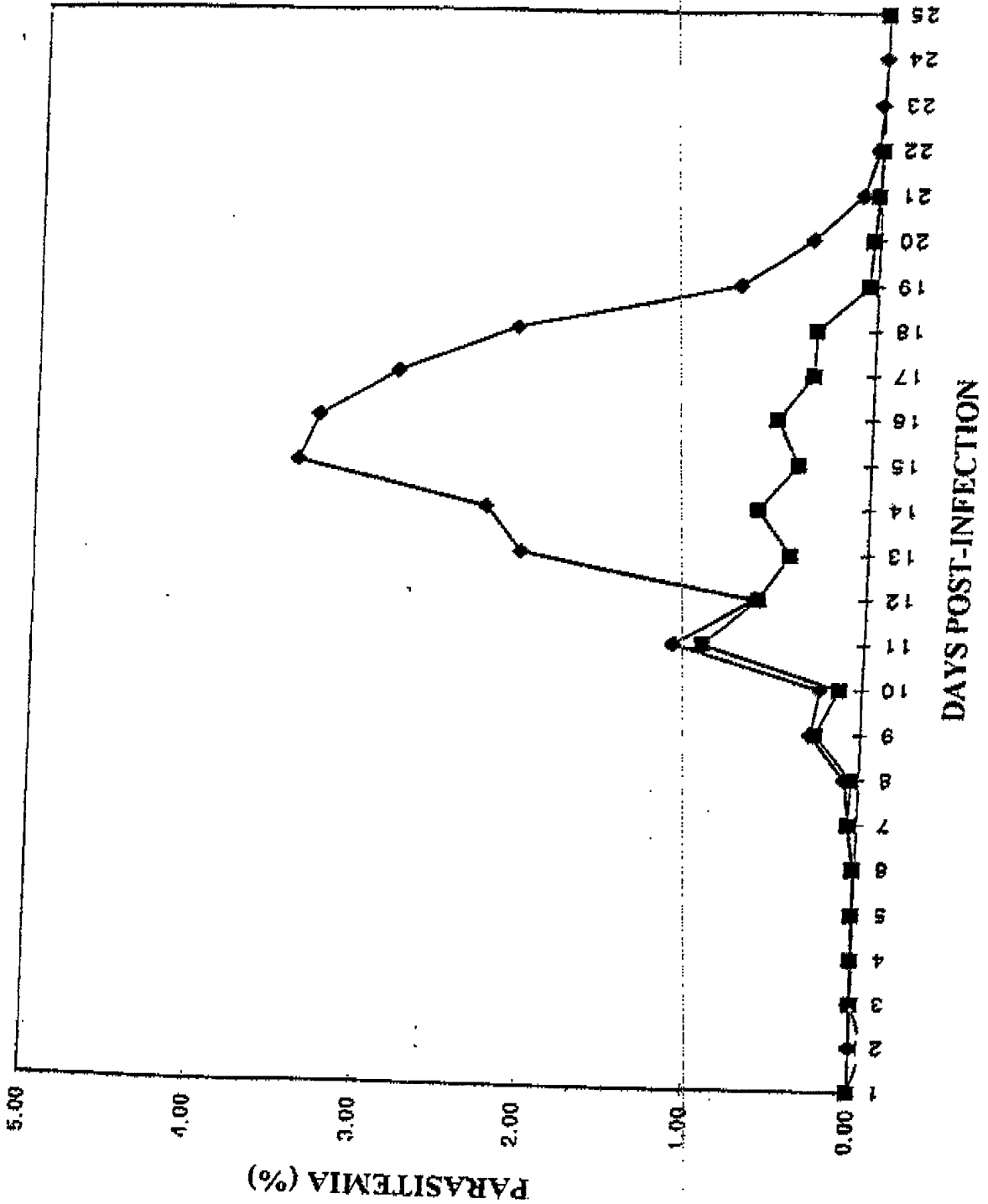
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Control-38  
Control-85

FIGURE 106

PLASMODIUM FALCIPARUM VACCINATION TEST IN SAIMIRI  
SCIUREUS SQUIRREL MONKEY  
PHYSIOLOGICAL WATER CONTROL





0660-0139-0X PCT

# Declaration, Power Of Attorney and Petition

Page 1 of 3

WE (I) the undersigned inventor(s), hereby declare(s) that:

My residence, post office address and citizenship are as stated below next to my name,

We (I) believe that we are (I am) the original, first, and joint (sole) inventor(s) of the subject matter which is claimed and for which a patent is sought on the invention entitled

RECOMBINANT PROTEIN CONTAINING A C-TERMINAL FRAGMENT OF  
PLASMODIUM MSP-1

the specification of which

- ☐ is attached hereto.
- ☐ was filed on \_\_\_\_\_ as  
Application Serial No. \_\_\_\_\_  
and amended on \_\_\_\_\_.
- ☒ was filed as PCT international application  
Number PCT/FR97/00290  
on February 14, 1997,  
and was amended under PCT Article 19  
on \_\_\_\_\_ (if applicable).

We (I) hereby state that we (I) have reviewed and understand the contents of the above-identified specification, including the claims, as amended by any amendment referred to above.

We (I) acknowledge the duty to disclose information known to be material to the patentability of this application as defined in Section 1.56 of Title 37 Code of Federal Regulations.

We (I) hereby claim foreign priority benefits under 35 U.S.C. § 119(a)-(d) or § 365(b) of any foreign application(s) for patent or inventor's certificate, or § 365(a) of any PCT International application which designated at least one country other than the United States, listed below and have also identified below, by checking the box, any foreign application for patent or inventor's certificate, or PCT International application having a filing date before that of the application on which priority is claimed. Prior Foreign Application(s)

Application No.	Country	Day/Month/Year	Priority Claimed
96/01822	France	14 February 1996	<input checked="" type="checkbox"/> Yes <input type="checkbox"/> No
_____	_____	_____	<input type="checkbox"/> Yes <input type="checkbox"/> No
_____	_____	_____	<input type="checkbox"/> Yes <input type="checkbox"/> No

We (I) hereby claim the benefit under Title 35, United States Code, § 119(e) of any United States provisional application(s) listed below.

(Application Number)

(Filing Date)

(Application Number)

(Filing Date)

We (I) hereby claim the benefit under 35 U.S.C. § 120 of any United States application(s), or § 365(c) of any PCT International application designating the United States, listed below and, insofar as the subject matter of each of the claims of this application is not disclosed in the prior United States or PCT International application in the manner provided by the first paragraph of 35 U.S.C. § 112, I acknowledge the duty to disclose information which is material to patentability as defined in 37 CFR § 1.56 which became available between the filing date of the prior application and the national or PCT International filing date of this application.

Application Serial No.

Filing Date

Status (pending, patented,  
abandoned)

PCT/FR97/00290

February 14, 1997

And we (I) hereby appoint: Norman F. Oblon, Registration Number 24,618; Marvin J. Spivak, Registration Number 24,913; C. Irvin McClelland, Registration Number 21,124; Gregory J. Maier, Registration Number 25,599; Arthur I. Neustadt, Registration Number 24,854; Richard D. Kelly, Registration Number 27,757; James D. Hamilton, Registration Number 28,421; Eckhard H. Kuesters, Registration Number 28,870; Robert T. Pous, Registration Number 29,099; Charles L. Gholz, Registration Number 26,395; Vincent J. Sunderdick, Registration Number 29,004; William E. Beaumont, Registration Number 30,996; Steven B. Kelber, Registration Number 30,073; Robert F. Gnusc, Registration Number 27,295; Jean-Paul Lavalleye, Registration Number 31,451; Stephen G. Baxter, Registration Number 32,884; Martin M. Zoltick, Registration Number 35,745; Robert W. Hahl, Registration Number 33,823; Richard L. Treanor, Registration Number 36,379; Steven P. Weihrouch, Registration Number 32,829; John T. Goolkasian, Registration Number 26,142; Marc R. Labgold, Registration Number 34,651; William J. Healey, Registration Number 36,160; Richard L. Chinn, Registration Number 34,305; Steven E. Lipman, Registration Number 30,044; Carl E. Schlier, Registration Number 34,426; James J. Kulbaski, Registration Number 34,648; Catherine B. Richardson, Registration Number 39,007; Richard A. Neifeld, Registration Number 35,299; J. Derek Mason, Registration Number 35,270; and Surinder Sachar 34,423; our (my) attorneys, with full powers of substitution and revocation, to prosecute this application and to transact all business in the Patent Office connected therewith; and we (I) hereby request that all correspondence regarding this application be sent to the firm of OBLON, SPIVAK, McCLELLAND, MAIER & NEUSTADT, P.C., whose Post Office Address is: Fourth Floor, 1755 Jefferson Davis Highway, Arlington, Virginia 22202.

We (I) declare that all statements made herein of our (my) own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issuing thereon.

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Signature of Inventor

Date 22 September 1998

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Date

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Date

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Date

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